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(54) Title: **HIGH DOSAGE PARENTERAL ADMINISTRATION OF LACTOFERRIN**

(57) Abstract: The invention provides methods of treatment using high dosages of lactoferrin. Lactoferrin can be administered par-  
enterally at high dosages without significant side effects to treat a variant of disorders including infectious diseases and inflammation.

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## High Dosage Parenteral Administration of Lactoferrin

### FIELD OF THE INVENTION

The present invention relates to the production, purification and use of recombinant human lactoferrin for parenteral administration in various therapeutic applications.

### BACKGROUND

Lactoferrin (LF) is a metal binding glycoprotein of Mr 77,000 found in milk, tears, saliva, bronchial, intestinal, vaginal and other secretions. LF is also present in the secondary granules of neutrophils. Lactoferrin plays an important role in numerous inflammatory and immune response functions such as regulation of monocyte colony stimulating factor synthesis, regulation of interleukin synthesis, activation of natural killer cell activity, inhibition of metastasis, and maturation of T-cells. Lactoferrin also inhibits myelopoiesis, binds to members of the low density lipoprotein receptor family, and blocks the clearance of lipoprotein chylomicron remnant particles (Sanchez, L. *et al.* (1992) *Arch Dis. Child.* 67:657, Iyer, S.*et al.* (1993) *Eur. J. Clin. Nutr.* 47:232, Huettinger, M.*et al.* (1992) *J. Biol. Chem.* 267:18551, Willnow, T.E. *et al.* (1992) *J. Biol. Chem.* 267:26172). It also appears to play a role in inhibiting the production or release of prostaglandin E<sub>2</sub>, interleukins, and tumor necrosis factor by mononuclear cells (Bartal, L. *et al.* (1987) *Pediatr. Res.* 21:54-57, Zucali, J.R.*et al.* (1989) *Blood* 74:1531, Crouch, S.P.M.*et al.* (1992) *Blood* 80:235).

Human LF (hLF) is also a major component of the non-specific defense of mucosal surfaces and neutrophils and is active against a variety of pathogens (reviewed in Nuijens, J.H.*et al.* (1996) *J. Mammary Gland Biol. Neoplasia.* 1:285 and Sanchez, L. *et al.* (1992) *Arch Dis. Child.* 67:657). This protein displays antimicrobial properties against Gram-positive and Gram-negative bacteria by limiting the availability of environmental iron (Bullen, J.J. (1981) *Rev. Infect. Dis.* 3:1127). However, since iron-saturated hLF is also able to kill certain bacteria (Ellison, R.T. (1994). *Adv. Exp. Med. Biol.* 357:71), mechanisms other than iron-depletion apparently are involved in the antibacterial activity of lactoferrin.

Some of the biological activities of LF may instead arise from its capacity to bind to other molecules. Direct intermolecular interactions between hLF and human lysozyme (hLZ) may explain the synergy between the antibacterial action of these two

proteins. Interaction of hLF with bacterial outer membrane components such as lipopolysaccharide (LPS) and porins presumably plays an important role in the antimicrobial activity of hLF. Binding of hLF to the lipid A portion of LPS inhibits the LPS priming of neutrophils for enhanced fMLP-triggered superoxide release. Interaction of LF with heparin may account for the neutralization of the anticoagulant activity of heparin.

Some biological activities of LF arise from interactions between LF and cells via membrane bound receptors. For example, LF binding to specific receptors on monocytes, macrophages and activated lymphocytes results in inhibition of cytokine production. Cells that exhibit specific binding to hLF include liver cells, intestinal cells, mammary gland epithelial cells, monocytic cell lines, activated lymphocytes, and platelets.

The amino acid sequence of LF has been determined by protein sequencing and sequencing of cDNA clones. hLF consists of a polypeptide chain of 692 amino acids. The amino terminal region of hLF contains two clusters of basic residues, RRRR (residues 2-5) and RNMRKVR (residues 25-31), whereas bovine LF (bLF) has only one cationic domain (residues 17-42 (Tomita, M. *et al.* (1991) *J. Dairy Sci.* 74:4137, Hoek, K.S. *et al.* (1997) *Antimicrob. Agents Chemother.* 41:54)). The LF polypeptide is folded into two globular lobes, each of which contains an iron-binding cleft. The high affinity of LF for iron confers to the protein certain antibacterial properties and, in addition, may play a role in the absorption of dietary iron by the small intestine.

There have been a number of studies to assess the therapeutic potential of human lactoferrin (see e.g., Boxer *et al.*, (1982) *J. Lab. Clin. Methods* 99, 866-872; Kurose *et al.*, (1994) *J. Leukoc. Biol.* 55, 771-777; Levergule *et al.*, *Eur. J. Biochem.* 213, 1205-1211 (1993)) These studies have indicated that at least in some circumstances, parenteral administration of lactoferrin results in significant side effects including neutropenia, intestinal mucosal injury and vascular leakage and tissue damage.

## DETAILED DESCRIPTION

### I. Introduction

The invention provides methods of treating human patients by parenteral administration of relatively high dosages of lactoferrin. The invention is premised, in part, on results that demonstrate that dosages of at least 60 mg/kg body weight of human lactoferrin can be safely administered to human patients (see Examples). These results are in contrast to the significant side effects reported in references discussed in the Background section. Although practice of the invention is not dependent on an understanding of mechanism, it is believed that the lack of side effects in the present methods results, in part, from the large amounts of human lactoferrin available to the present inventors through the production of lactoferrin in milk of transgenic cattle, and the consequent ease of purification of pharmaceutical grade lactoferrin for parenteral administration. The ability safely to administer high dosages is advantageous in a number of therapeutic applications. Intact LF and fragments or variants of LF are used at high doses, with a lack of adverse effects, to treat diseases and conditions that require a bolus of and/or sustained large doses. Some diseases and conditions that can be treated are gastroenteritis, Inflammatory Bowel Diseases, sepsis, ARDS, MOF, GVD, GVHD and systemic inflammation. Large doses, in the absence of adverse effects, allow the blocking or neutralizing of inflammatory agents and/or boost the clearance of inflammatory agents. IBD (ulcerative colitis and Crohn), for instance is known to cause leakage of the gut allowing LPS or other infectious agents to pass through into the blood system. Administering hLF can block these free LPS or other infectious or inflammatory agents and cause them to clear from the body more rapidly and/or to mask their inflammatory activity, thus rendering them harmless. By forming a complex with hLF, inflammatory agents are cleared more quickly through the liver. Additionally, by increasing membrane permeability, hLF assists the passage of antibiotics, thus potentiating their antibacterial effects (see Kuipers et al., *Antimicrobial Agents and Chemotherapy* 43, 2635-2641).

hLF is useful in a variety of therapeutic and prophylactic applications, including use as antimicrobial agents and the treatment of, *e.g.*, inflammation, anemia, myelopoieses and for reducing reperfusion injury, cytokine release, and proteoglycan-mediated entry of virus into cells. LF variants are also useful for treatment of these diseases and conditions, and are especially useful for treatment of those conditions for which beneficial effects of natural LF treatment are due to binding to a high affinity LF receptor. Such LF variants have the biological activities of natural LF, *e.g.*, binding to high affinity LF receptors on

cells, but with reduced binding, relative to natural LF, to heparin, DNA, human lysozyme, the Lipid A component of bacterial lipopolysaccharide (LPS), and sulfated cell surface molecules. Thus, an advantage to the use of the LF variants is that the desired physiological effect can be achieved while avoiding side effects caused by the binding of natural LF to heparin, DNA, human lysozyme, Lipid A, or cell surface proteoglycans. For example, the LF variants can be used to deliver nutritional iron to cells, without concurrent neutralization of heparin and similar effects. Because some LF variants have little or no binding to sulfated cell surface molecules, and bind with increased affinity to high affinity LF receptors, more efficient targeting of LF to these receptors can be achieved.

Such variants include short polypeptides having one or more arginines in the N-terminal segment of the polypeptide, such as found in the first cationic domain of hLF, which have been found to exhibit significant therapeutic activity. Other variants of LF have 1-4 arginine residues from the first basic cluster (*i.e.*, residues 2-5) deleted. Some variants include one or more residues from the first cationic domain of hLF, but not amino acids from the second cationic domain. Some polypeptides are quite short, such as less than 27 amino acids in length. Given their short length, such polypeptides are easily and inexpensively prepared and are readily amenable to use in pharmaceutical compositions.

## II. Definitions

The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. Unless otherwise stated, the term also applies to amino acid polymers in which one or more amino acids are chemical analogues of a corresponding naturally occurring amino acid.

The term lactoferrin protein refers generically to intact lactoferrin proteins found in nature, allelic, species and induced variants thereof, and fragments. Induced variants typically show at least 85% amino acid sequence identity over the entire length of the variant to a natural lactoferrin sequence when maximally aligned using the BLASTN algorithm with a wordlength (W) of 3, M=5, and N= -4 (see Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990)). Variants preferably differ predominantly or exclusively by conservative mutations from a natural form of human lactoferrin. Fragments typically comprise at least 6, 10, 20, 50, 100 or 200 contiguous amino acids from a natural lactoferrin sequence. Examples of natural human lactoferrin sequences are provided in

SEQ ID NO:1, by Powell, M.J. and Ogden, J.E., Nucleic Acids Res. 18:4013 (1990), which is incorporated herein by reference, Rado, T.A., *et al.*, Blood 70:989-993 (1987), and Heyneker, H.L., WO 91/08216, each of which is incorporated herein by reference.

Some lactoferrin proteins have a modified protein backbone. Examples of such

5 modifications include acetylations, carboxylation, glycosylation modifications and other processing variants of hLF. Some lactoferrin variants comprise polypeptides having the sequence of natural LF from which 1, 2, 3 or 4 arginine residues at the amino terminus have been removed (*i.e.*, deletion of all or part of the first basic cluster) or from which the residues of the second basic cluster have been removed, or from which both the first and  
10 second basic clusters have been removed. Other LF variants have a deletion of the second basic cluster and deletions of one or more amino-terminal arginine residues. Still other LF variants are hLF from which the residues of the second basic cluster have been mutated (*e.g.*, to uncharged residues). The amino-terminal sequence of hLF is: N'-GRRRSVQWC.

Some LF variants include a variant having a deletion of one arginine (along with the  
15 terminal glycine) residue (referred to as hLF-2N), a variant having two arginine residues removed (referred to as hLF-3N), a variant having three arginine residues removed (referred to as hLF-4N), and a variant having all four arginine residues removed (referred to as hLF-5N). The arginine residues of the first basic cluster can be removed by proteolysis of natural LF or by expression of a polynucleotide encoding a truncated hLF.  
20 Alternatively, one or more arginine residues of the first basic cluster can substituted for by other (*i.e.*, other than arginine) amino acids, *e.g.*, by directed mutagenesis of a polynucleotide encoding hLF. In some variants, one or more arginine residues of the first basic cluster are deleted or mutated to an amino acid that is not positively charged at physiological pH, *i.e.*, a neutral or acidic residue, usually to a neutral amino acid, most  
25 often alanine, leucine, glycine, valine or isoleucine. Hereinafter, reference to a hLF variant from which all or some of the arginine residues the first basic cluster have been "deleted" or "removed" refers both to removal of the arginines of the first basic cluster by deletion or by mutagenesis, unless stated otherwise.

The term "naturally-occurring" as applied to an object refers to the fact that an  
30 object can be found in nature. Natural lactoferrin includes recombinantly encoded hLF ("rhLF") expressed in a transgenic non-human animal, such as a bovine, where the glycosylation pattern may be distinct from glycosylation patterns of naturally occurring hLF obtained from human milk. Natural hLF includes recombinantly encoded hLF expressed in a transgenic non-human animal, such as a bovine, where the glycosylation

pattern may be distinct from glycosylation patterns of naturally occurring hLF obtained from human milk.

"Neutralized lactoferrin" is LF having substantially the sequence of native LF but that, by virtue of modification of the residues of the first basic cluster, is not able to bind to a LF ligand, *e.g.*, heparin, as measured by solid phase ligand binding assay, but still binds 105 kD LF receptor found on Jurkat human lymphoblastic T-cells (Bi *et al.* (1994) *Eur. J. Cell Biol.* 65:164 and Bi *et al.* (1996) *Eur. J. Cell Biol.* 69:288). "Modification" includes chemical modification of the residues of the first basic cluster or, alternatively, binding of a molecule that blocks (*i.e.*, through steric hinderance) the interaction of the first basic cluster of LF and heparin. Blocking molecules include monoclonal antibodies, fragments thereof, and LF ligands such as human lysozyme or heparin.

In a shorthand format for referring to subsequences of hLF, the specific residues being referred to are placed in parentheses. For example, hLF(1-11) refers to residues 1 to 11 inclusively from the N-terminus of hLF; similarly, hLF(2-11) refers to residues 2 to 11 inclusively from the N-terminal region of hLF. hLF (*i.e.*, the full-length protein) that lacks a certain number of residues from the N-terminus is referred to as hLF<sup>-xN</sup>, where x is the number of N-terminal residues missing. Thus, for example, hLF in which the N-terminal glycine and arginine are removed is referred to as hLF<sup>-2N</sup>; hLF missing the N-terminal glycine and the two N-terminal arginines, is referred to as hLF<sup>-3N</sup>, and hLF lacking the N-terminal glycine and first three arginine residues is referred to as hLF<sup>-4N</sup>. The three arginines located at the N-terminus (*i.e.*, residues 2, 3 and 4 of SEQ ID NO:1) are referred to as Arg<sup>2</sup>, Arg<sup>3</sup> and Arg<sup>4</sup>, respectively. Unless otherwise stated, the N-terminal amino acid of hLF refers to Gly<sup>1</sup> (see SEQ ID NO:1); the 31 residues located at the amino-terminus of hLF are: N'-GRRRRSVQWCAVSQPEATKCFQWQRNMRKVR (residues 1-31 of SEQ ID NO:1).

A "conservative substitution," when describing a protein, refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well-known in the art. See, *e.g.*, Creighton (1984) *Proteins*, W.H. Freeman and Company. In addition, individual

substitutions, deletions or additions, which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations."

The phrases "specifically binds to a protein" or "specifically immunoreactive with," when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, a specified antibody binds preferentially to a particular protein and does not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A molecule such as an antibody that specifically binds to a protein has an association constant of at least  $10^6 \text{ M}^{-1}$  or  $10^7 \text{ M}^{-1}$ , preferably  $10^8 \text{ M}^{-1}$  to  $10^9 \text{ M}^{-1}$ , and more preferably, about  $10^{10} \text{ M}^{-1}$  to  $10^{11} \text{ M}^{-1}$  or higher. A variety of immunoassay formats can be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See, e.g., Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

Intact human lactoferrin, its fragments and variants are "substantially free" of other human proteins when at least about 90%, more usually at least about 95%, and most commonly at least about 99% of the *human* protein present in the sample is the human lactoferrin, fragment or variant. The amount of any specific protein present in a sample can be determined by quantitative SDS-PAGE (for relatively simple mixtures) or by immunological assays (e.g., ELISA and RIA) for more complex mixtures (e.g., a mixture of bovine milk proteins and LF variant).

"Substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition comprises more than about 80 to 90 percent, and preferably 95%, 99%, 99.5% or 99.9% of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the



composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

Immunological and molecular biological methods are well known and are described, for example, in Sambrook *et al.*, Molecular Cloning - A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 and Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York (1988), both of which are incorporated herein in their entirety and for all purposes.

The term "patient" includes human and veterinary subjects.

### 10 III. Production of lactoferrin and lactoferrin polypeptides and variants

#### A. Purification and production of LF

LF is abundant in milk and is most easily purified from this source, although it is also found in exocrine secretions and secondary granules of neutrophils. A preferred source of hLF is milk from a transgenic bovine species containing a hLF transgene (see section III, C). The transgene-encoded hLF is substantially purified from other milk proteins in the milk of transgenic cows, and is preferably substantially isolated from endogenous bovine LF, if present in the milk. Numerous methods for purification of hLF from milk have been reported. See, for example, U.S. Patents 4,436, 658; 4,791,193; and 4,668,771, which are incorporated herein by reference. See also, Nuijens *et al. J.*, 1996, *Mammary Gland Biology and Neoplasia* 1:3, 283-293 (1996) and references cited therein.

A preferred method for hLF purification is described PCT Application PCT/EP95/00583, which is incorporated herein by reference. Briefly, milk or a milk fraction containing hLF is contacted with a strong cation exchange resin (e.g., S Sepharose™) in the presence of relatively high ionic strength (0.2M to 0.5M NaCl or KCl, preferably 0.4M NaCl or KCl) to prevent binding of non-LF proteins and other substances to the strong cation exchange resin and to reduce electrostatic interactions of LF with other proteins (e.g., caseins) or substances (e.g., lipopolysaccharide), and to liberate LF from complexes. The strong cation exchange resin containing the bound LF is separated from the unbound compounds in the milk or milk fraction, typically by centrifugation or sedimentation followed by batchwise washing and/or by pouring the resin into a column and washing the beads with buffer having approximately equal or lower salt concentration. The LF bound to the cation exchange resin is eluted with an aqueous, typically buffered, NaCl or KCl gradient (e.g., linear gradient of 0-1M NaCl in 20 mM sodium phosphate, pH 7.5) or by batch elution or stepwise elution with an

aqueous, preferably buffered, NaCl or KCl solution of 0.4M or greater, preferably at least 0.5M NaCl or KCl. By selecting appropriate elution conditions, hLF can be substantially purified from bovine milk and substantially separated from bovine LF by an efficient method.

5 hLF (e.g., rhLF) can be further purified from endogenous LF (e.g., bLF) by the additional subsequent step of rechromatography on a strong cation exchanger, such as S Sepharose™ Fast Flow, with salt gradient or stepwise elution to separate hLF from remaining traces of endogenous non-human LF species (e.g., bLF), and/or can optionally include affinity chromatography with a concanavalin A resin to further separate hLF from  
10 bLF, with bLF being more strongly bound to the Con A resin than hLF.

A modified and improved procedure for the purification of hLF from milk is described in the Example under (II) Methods. Briefly, milk or a milk fraction, preferably skimmed milk, is contacted with a strong cation exchanger, preferably S Sepharose. The strong cation exchange resin containing the bound LF is washed with a buffer containing  
15 at least about 0.1 M salt, preferably NaCl, and then washed with a buffer containing at least about 0.4 M salt, preferably NaCl. After washing, the bound LF is eluted from the strong cation exchanger using a buffer containing at least 1.0 M salt, preferably NaCl, and concentrated and desalted to about 0.1 M of the salt. Optionally, at this stage the LF preparation is further microfiltrated and subjected to virus inactivation by adding a non-  
20 ionic surfactant, such as Tween 80 (at about 1% w/v), and a di- or trialkylphosphate, such as tri-n-butyl phosphate (at about 0.3% w/v), and incubating for at least 10 hours at a temperature between 15°C and 30°C, preferably about  $25 \pm 1^\circ\text{C}$ . The (virus inactivated) LF sample is then loaded onto a Q Sepharose column serially connected with a Macro-  
25 prep High S Support (MPHS, Biorad) column, both equilibrated in a buffer with about 0.1 M salt, preferably NaCl. The columns are washed with a buffer containing 0.1 M salt, preferably NaCl. The Q Sepharose column is then disconnected and the MPHS column is washed extensively with low salt buffer (e.g. 20mM sodium phosphate at pH 7.5). The MPHS column is stepwise eluted with first a buffer with about 0.4 M salt and then with a buffer with at least 0.5 M salt, preferably NaCl. Eluted fractions are tested for  
30 hLF (e.g., rhLF) content and endogenous LF (e.g. bLF) impurity. rhLF fractions free of endogenous LF (bLF) are pooled, concentrated, filtered over a 15 nm filter, concentrated, buffer exchanged to saline (0.9% NaCl), sterile filtrated (0.1  $\mu\text{m}$ ) and stored at  $< -65^\circ\text{C}$ .

## B. Production of variants and polypeptide fragments of LF

### i. Polypeptides and variants

The present methods use intact lactoferrin and fragments and variants thereof. Some methods employ various polypeptides which include at least one or more arginines from the first cationic domain of hLF (residues 2-5 of SEQ ID NO:1), but which exclude the residues which make up the second cationic domain (residues 28-31 of SEQ ID NO:1). Thus, for example, some polypeptides comprise at least 6 but no more than 27 contiguous amino acids from the N-terminal segment of hLF (SEQ ID NO:1), wherein the N-terminus of the polypeptide is residue 1 of SEQ ID NO:1, *i.e.*, glycine.

Some methods employ polypeptides that include the first cationic domain but not the second cationic domain of hLF, and that also lack one or more residues from the N-terminus of hLF. For example, some methods of the invention use polypeptides comprising at least 6 amino acids but no more than 26 contiguous amino acids from hLF, wherein the N-terminus of the polypeptide is Arg<sup>2</sup> (for example, a hLF fragment lacking Gly<sup>1</sup>). Some methods use polypeptides comprising at least 6 but no more than 25 contiguous amino acids from the N-terminal segment of hLF, wherein the N-terminus of the polypeptide is Arg<sup>3</sup> (for example, a hLF fragment lacking Gly<sup>1</sup>Arg<sup>2</sup>).

In some instances, the hLF polypeptide fragments just described are chosen so that cysteine residue 10 (see SEQ ID NO:1) is retained. In other cases, the polypeptide includes a cysteine at approximately the same location in the sequence. Such a cysteine can be used to dimerize one fragment with another polypeptide having a cysteine residue, such as another hLF fragment, for example. In some instances, dimerization can increase the activity of the polypeptide.

Some fragments include more than 6 contiguous amino acids from the N-terminus of hLF. For example, some polypeptides include 7, 8, 9, 10, 11 or 12 contiguous amino acids from hLF, for example. Some polypeptides include fewer than 27, 26 or 25 contiguous amino acids of hLF. Some polypeptides include less than 24, 22, 20, 18, 16, 14 or 12 contiguous amino acids from hLF, or any number of amino acids therebetween. Some polypeptides include no more than 19 contiguous amino acids from hLF. Some polypeptides include no more than 11 contiguous amino acids from hLF, for example, hLF(1-11), hLF(2-11) and hLF(3-11).

Some fragments of lactoferrin contain at least residues 1-90 of lactoferrin (designated N-terminal domain I). This domain has been shown to bind specifically to phytohemagglutinin-stimulated peripheral blood human lymphocyte receptors (see

Rochard et al., Fed. Eur. Biochem. Soc. 1, 201-204 (1989). Some fragments contain at least residues 1-47. Such fragments have been reported to show bacterial activity (see Kiwata et al., Biochem. Biophys. Res. Commun. 245, 764-3 (1998)).

5                    ii. Preparation

LF variants lacking one or more of the amino terminal arginine residues can be produced by a variety of methods. Preferred methods of production include (a) proteolytic cleavage of natural LF, (b) synthesis, or (c) recombinant expression, e.g., mutagenesis of a LF gene followed by expression in cells or transgenic animals of the LF variant, with recombinant expression most preferred. Deletion of the residues of the second basic cluster is preferably carried out by *in vitro* mutagenesis.

a. Proteolysis

LF variants can be produced by cleavage of purified LF with a protease, preferably a serine protease and most preferably trypsin.

The tryptic digestion of purified natural LF can be carried out as follows: Five milligrams of native hLF are incubated with trypsin at an enzyme: substrate molar ratio of 1:8 at 37°C in PBS. Digestion is stopped after 1, 5, 25 min and 3 h by the addition of a 12-fold molar excess of SBTI and N-terminal integrity is monitored, for example by analytical Mono S chromatography (Van Berkel *et al.* (1995) *Biochem. J.* 312:107) and standard techniques such as SDS-PAGE, chromatography, and protein sequencing.

Following proteolysis, the LF variants can be separated from each other and from natural (*i.e.*, uncleaved) hLF (and other proteins, if present) by cationic exchange chromatography (e.g., Mono S; heparin), Hydrophobic Interaction Chromatography (HIC) or Cibracon Blue Sepharose chromatography. LF variants can be separated from uncleaved LF (and each other) by batch-wise incubation of recombinantly expressed LF or LF variants and S Sepharose for 4 h. The mixture is poured into a column and the LF eluted with 20 mM sodium phosphate, 0.5 M NaCl, pH 7.5. The S Sepharose eluate is diluted in 20 mM sodium phosphate, pH 7.5 (buffer A), applied on a Mono S HR 5/5 cation exchange column and eluted with a linear salt gradient of 0 to 0.5 M NaCl in 60 ml of buffer A at a flow rate of 0.5 ml/min. Natural hLF elutes at 0.7 M NaCl (Van Berkel *et al.* (1995) *Biochem. J.* 312:107) and hLF-5N elutes at about 0.33 M NaCl. The hLF-3N and hLF-2N species elute from Mono S at about 0.5 and about 0.6 M NaCl, respectively.

Certain polypeptides used in the present methods can also be prepared via a

reduction and proteolysis method. This approach begins with pepsin digestion of hLF according to known methods (see, e.g., Bellamy, W. *et al.*, (1992) *Biochim. Biophys. Acta.* 1121:130; and Tomita, M. *et al.*, (1991) *J. Dairy Sci.* 74:4137). In order to break the disulfide bond between Cys 10 and Cys 46, the digested products are subsequently reduced and alkylated using standard reagents (e.g., DTT or  $\beta$ -mercaptoethanol for reduction and iodoacetamine or 4-vinylpyridine for alkylation) according to known methods (see, e.g., "Current Protocols in Protein Chemistry," Coligan, J.E., *et al.*, Eds. John Wiley and Sons, Inc). The order can be reversed so that the reduction and alkylation steps precede the pepsin digestion step. After digestion, reduction and alkylation, N-terminal peptides can be isolated from the digestion mixture by standard chromatographic methods, including for example, cation exchange, gel filtration, HIC or RP-HPLC. Further useful hLF variants can be obtained by proteolytic cleavage of purified intact hLF using one or more proteases, such as bromelain, cathepsin B, cathepsin D, cathepsin G, chymotrypsin, clostripain, elastase, endoproteinase-Arg-C, endoproteinase-Asp-N, endoproteinase-Glu-C, endoproteinase-Lys-C, Factor Xa, papain, pepsin, proteinase K, subtilisin, thermolysin and trypsin. The above proteases are commercially available.

Intact lactoferrin can also be converted to fragments by a variety of mechanisms in vivo. For example, lactoferrin can be degraded by release of elastase (a serine protease) as a result of neutrophil degranulation (Nuijens *et al.*, *J. Lab. Clin. Med.* 119, 159-168 (1992). Lactoferrin can also be degraded by bacterial derived protease (see Britigan *et al.*, *J. Clin. Invest.* 88, 1092-102 (1991), by membrane bound proteases (see Birgens *et al.* *Eur. J. Haematol.* 45, 31-35 (1990) and as a result of pepsin degradation in the stomach (see Kuwata *et al.*, *Biochem. Biophys. Res. Comm.* 245, 764-73 (1998). Fragments of the type produced by in vivo degradation of intact lactoferrin, for example, at sites of inflammation or infection, are also particularly useful agents for direct administration. Such fragments can be produced in vitro by proteolytic cleavage of intact hLF, using one or more of the above-mentioned commercially available endoproteases. Preferentially, the in vitro produced hLF derived peptides are purified using conventional means before formulating as a pharmaceutical composition for administration..

#### b. Synthesis

Fragments of lactoferrin can be synthesized by the well-known Merrifield solid-phase synthesis method in which amino acids are sequentially added to a growing chain. See Merrifield (1963), *J. Am. Chem. Soc.* 85:2149-2156; and Atherton *et al.*, "Solid Phase

Peptide Synthesis," IRL Press, London, (1989). Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, California.

5                                    c. Recombinant techniques

Alternatively, intact lactoferrin and fragments and variants thereof can be prepared using recombinant techniques in which a nucleotide sequence encoding the polypeptide of interest is expressed in cultured cells such as described in Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York  
10 (1987) and in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, 2nd ed., vols. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989) both of which are incorporated herein by reference in their entirety. Also see, Kunkel (1985) *Proc. Natl. Acad. Sci.* 82:488 (describing site directed mutagenesis) and Roberts *et al.* (1987) *Nature* 328:731-734 or Wells, J.A., *et al.* (1985) *Gene* 34:315 (describing cassette  
15 mutagenesis).

Typically, nucleic acids encoding the desired polypeptides are used in expression vectors. The phrase "expression vector" generally refers to nucleotide sequences that are capable of affecting expression of a gene in hosts compatible with such sequences. These expression vectors typically include at least suitable promoter sequences and optionally,  
20 transcription termination signals. Additional factors necessary or helpful in effecting expression can also be used as described herein. DNA encoding a polypeptide is incorporated into DNA constructs capable of introduction into and expression in an *in vitro* cell culture. Specifically, DNA constructs are suitable for replication in a prokaryotic host, such as bacteria, *e.g.*, *E. coli*, or can be introduced into a cultured  
25 mammalian, plant, insect, *e.g.*, Sf9, yeast, fungi or other eukaryotic cell lines.

DNA constructs prepared for introduction into a particular host typically include a replication system recognized by the host, the intended DNA segment encoding the desired polypeptide, and transcriptional and translational initiation and termination regulatory sequences operably linked to the polypeptide encoding segment. A DNA  
30 segment is "operably linked" when it is placed into a functional relationship with another DNA segment. For example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence. DNA for a signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide. Generally, DNA sequences that are

operably linked are contiguous, and, in the case of a signal sequence, both contiguous and in reading phase. However, enhancers need not be contiguous with the coding sequences whose transcription they control. Linking is accomplished by ligation at convenient restriction sites or at adapters or linkers inserted in lieu thereof.

5       The selection of an appropriate promoter sequence generally depends upon the host cell selected for the expression of the DNA segment. Examples of suitable promoter sequences include prokaryotic, and eukaryotic promoters well-known in the art. *See, e.g.,* Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2d ed.), vols. 1-3, Cold Spring Harbor Laboratory (1989). The transcriptional regulatory sequences typically  
10       include a heterologous enhancer or promoter which is recognized by the host. The selection of an appropriate promoter depends upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known and available. *See* Sambrook *et al.*, *supra*. Expression vectors include the replication system and transcriptional and translational regulatory sequences together with the  
15       insertion site for the polypeptide encoding segment can be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook *et al.*, *supra*, and in Metzger *et al.* (1988) *Nature* 334:31-36. For example, suitable expression vectors can be expressed in, *e.g.*, insect cells, *e.g.*, Sf9 cells, mammalian cells, *e.g.*, CHO cells and bacterial cells, *e.g.*, *E. coli*.

20       *In vitro* mutagenesis and expression of mutant proteins are described generally in Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York (1987) and in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989, both of which are incorporated herein by reference in their entirety and for all  
25       purposes. Also see, Kunkel (1985) *Proc. Natl. Acad. Sci.* 82:488 (describing site directed mutagenesis) and Roberts *et al.* (1987) *Nature* 328:731-734 or (Wells, J.A., *et al.* (1985) *Gene* 34:315 (describing cassette mutagenesis).

Another method for preparing polypeptides is to employ an *in vitro* transcription/translation system. DNA encoding a polypeptide is cloned into an  
30       expression vector as described *supra*. The expression vector is then transcribed and translated *in vitro*. The translation product can be used directly or first purified. Polypeptides resulting from *in vitro* translation typically do not contain the post-translation modifications present on polypeptides synthesized *in vivo*. Methods for synthesis of polypeptides by *in vitro* translation are described by, for example, Berger &

Kimmel, *Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques*, Academic Press, Inc., San Diego, CA, 1987 (incorporated herein by reference in its entirety).

5            C. Transgenic animals

     In certain instances, hLF or hLF polypeptides or variants are produced by expression in transgenic animals (*i.e.*, non-human animals containing an exogenous DNA sequence in the genome of germ-line and somatic cells introduced by way of human intervention) such as bovines, goats, rabbits, sheep, pigs or mice. Methods for production of recombinant polypeptides by transgenic non-human species are known in the art and are described, for example, in U.S. Patent Nos. 5,304,489; 5,633,076; and 5,565,362 which are incorporated herein by reference in their entirety, as well as in PCT publications PCT/US93/05724 and PCT/US95/09580, both of which are incorporated herein by reference in their entirety. An advantage of the transgenic animals is the isolation of hLF, variants and polypeptides of interest in large amounts, especially by economical purification methods. For example, the production of transgenic bovine species containing a transgene encoding a human LF polypeptide targeted for expression in mammary secreting cells is described in WO 91/08216, incorporated herein by reference in its entirety. When LF variants are produced in transgenic bovines the human protein typically is separated from the bovine milk proteins (*e.g.*, whey proteins, caseins, bovine LF, IgA, albumin, lysozyme,  $\beta$ -lactoglobulin) before use (*e.g.*, administration to patients). Alternatively, use can be made of whole or partially purified bovine milk containing the desired hLF protein, variant or polypeptide.

25           D. Alternative Methods for Neutralizing hLF Basic Clusters

     Although deletion of the residues in the first or second basic cluster of hLF is a preferred method for generating a hLF with changed physiological properties, other methods for neutralizing one or both basic clusters exist. For example, the first basic cluster can be neutralized by incubating hLF with ligands such as heparin, which binds at the first cluster and inhibits binding of LF to the 105kd LF receptor, LPS, hLZ, and other molecule for which binding is first-cluster dependent.

     A preferred method for neutralizing the first basic cluster is to incubate hLF with a monoclonal antibody that binds at the amino terminus and prevents binding between the



first basic cluster and a target molecule (*e.g.*, heparin). Methods for producing monoclonal antibodies are well known (see, *e.g.*, Goding *et al.*, Monoclonal Antibodies: Principles and Practice (2d ed.) Acad. Press, N.Y., and Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988). Use of human or humanized monoclonal antibodies are most preferred because this reduces the possibility of an antigenic response following administration to a patient (see *e.g.*, U.S. Patent Nos. 5,569,825 and 5,585,089). Antigen-binding fragments of monoclonal antibodies, *e.g.*, Fab, Fab' F(ab')<sub>2</sub>, Fabc and Fv fragments, are similarly useful. Antibodies or antibody fragments can also be used for binding to the second basic cluster and preventing second-cluster dependent binding.

#### IV. Properties of lactoferrin and lactoferrin variants

##### A. Binding to heparin, Lipid A, DNA and human lysozyme

Natural hLF binds to heparin, Lipid A, DNA and hLZ. LF variants lacking one, two or three arginines of the first basic cluster show a strong decrease in reactivity for each of these four ligands, and binding is undetectable in standard assays (*e.g.*, solid phase ligand binding assays) when all four of the amino-terminal arginine residues are deleted.

The binding properties of natural LF and the LF variants can be measured in standard binding assays (see next section) and expressed in terms of *reactivity* where the level of binding of natural LF to a ligand is defined as 100% reactivity. The reactivity of the LF variants with the natural LF ligands heparin, DNA, Lipid A and hLZ is typically less than 80%, more usually less than 60%, often less than 15%. For some LF variants (*e.g.*, those with a deletion of all four arginine residues from the first basic cluster) reactivity is undetectable.

##### B. Binding assays

Assays suitable for measuring ligand-receptor interactions, such as the binding by natural LF and LF variants to heparin, lipid A, DNA, and hLZ, include assays solid-phase ligand binding assays and competitive solid-phase binding assays (see, *e.g.*, Mann *et al.* (1994) *J. Biol. Chem.* 269:23661-67). In some methods, the solid-phase binding assays measure binding by LF variants and natural LF. Typically, binding of the LF receptor by hLF or a hLF variant results in activation of the LF receptor. Methods for assaying receptor activation are known, for example, the resulting intracellular calcium shift can be measured (see, *e.g.*, Misra *et al.* (1994) *J. Biol. Chem.* 269:18303-306).

### C. Specific binding to cell membrane associated receptors

Some of the biological activities of hLF are linked to its ability to strongly chelate iron, whereas other activities relate to the interaction of hLf with target cells, including  
 5 intestinal cells (Hu *et al.* (1990) *Biochemistry* 29, 535-541; Kawakam *et al.* (1991) *Am. J. Physiol.* 261, G841-G846; Mikogami *et al.* (1994) *Am. J. Physiol.* 267, G308-G31),  
 mammary gland epithelial cells (Rochard *et al.* (1992) *Anticancer Res.* 1, 2047-2052),  
 hepatocytes (Regoeczi *et al.* (1985) *Am. J. Physiol.* 248, G8-G14; MacAbee *et al.* (1991)  
*J. Biol. Chem.* 226, 23624-23631; Ziere *et al.* (1992) *J. Biol. Chem.* 267, 11229-11235),  
 10 monocytes (Ismail *et al.* (1993) *J. Biol. Chem.* 268, 21618-21625), activated lymphocytes  
 (Mazurier *et al.* (1989) *Eur. J. Biochem.* 179, 481-487) and platelets (Leveugle *et al.*,  
 (1993) *Eur. J. Biochem.*, 213,1205-1211) each of which is incorporated by reference in  
 their entirety and for all purposes.

LF binds to cell surfaces through two classes of LF binding sites: relatively low  
 15 affinity sites which are cell surface sulfated molecules (*e.g.*, cell surface proteoglycans or  
 glycosaminoglycans) and high affinity receptors. Binding to the low affinity sites is  
 mediated by the first cluster of basic arginine residues, and deletion (or neutralization) of  
 one or more of these residues reduces or eliminates binding to the low affinity sites. Thus,  
 an hLF variant typically binds a high affinity LF receptor with an affinity of at least about  
 20 10 nM, usually between about 10 nM and about 40 nM. Cell binding assays are well  
 known and are described in, *e.g.*, Mazurier (1989) *Eur. J. Biochem.* 179:481-87. In  
 contrast, deletion of one or more of the amino-terminal arginine residues does not reduce  
 or abolish binding to the high affinity LF receptor.

High affinity LF binding sites have been found on activated lymphocytes,  
 25 mammary gland epithelial cells, platelets, monocytes, macrophages, intestinal cells, and  
 hepatocytes and are thought to exist on other cell types as well. A 105 kD specific hLF  
 receptor has been characterized in activated lymphocytes (Mazurier *et al.* (1989) *Eur. J.*  
*Biochem.* 179, 481-487), the Jurkat T-cell line (Bi *et al.* (1994) *Eur. J. Cell Biol.* 65, 164-  
 171; Bi *et al.* (1996) *Eur. J. Cell Biol.* 69, 288-296) and platelets (Leveugle *et al.* (1993)  
 30 *Eur. J. Biochem.*, 213,1205-1211).

Binding of LF to the 105 kD receptor has been shown to inhibit platelet aggregation  
 and is likely involved in the growth factor and/or differentiation activities of hLF. This  
 receptor has been localized in human lymphoblastic T-cells (*i.e.*, Jurkat cells, Pawelec *et al.*  
*(1982) Eur. J. Immuno.* 12:387-92) to the cell surface in coated pits vesicles as well as

in intracellular vesicles. Internalization of hLF by Jurkat cells has been demonstrated. Jurkat cells can be obtained from the American Tissue Type Collection American Type Culture Collection [ATCC] located at 12301 Parklawn Dr., Rockville, Maryland, USA 20852. LF binds to the lymphocyte (Jurkat cell) high affinity receptor with a  $K_D$  of approximately 40 nM.

The 105 kD receptor can be identified by immunological methods. For example, a specific rabbit anti-105 kD receptor polyclonal antibody has been described. This, or a similar polyclonal antibody, or an anti-105 kD receptor monoclonal antibody, can be used to identify the receptor on other cell types. For example, the polyclonal antibody referred to *supra* has been found to bind to epithelial cells from non-malignant human breast, benign mastopathies and breast carcinomas (Rochard *et al.* (1992) *Anticancer Research* 12: 2047-52). Alternatively, the 105 kD receptor can be identified by ligand blotting (see, *e.g.*, Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York) using labeled hLF (or amino-terminally deleted hLF) and membrane protein preparations of cells.

A specific hLF receptor has been isolated from intestinal brush border membranes and has a reported  $M_r$  of 110 (Kawaskami and Lonnerdal (1991) *Am. J. Physiol.* 261:G841-46). It is likely, but has not yet been demonstrated, that this receptor is the same as, or closely related to, the 105 kD receptor.

In hepatocytes, LF binds to a chylomicron remnant receptor or the Low density lipoprotein Receptor-related Protein (LRP) present on the cell surface. LF inhibits uptake of beta-VLDL containing chylomicron remnants. LF binding to murine peritoneal macrophages apparently occurs via the LRP, a member of the structurally related cell surface receptor family that mediates endocytosis of lipoproteins and other plasma proteins. The nature of hLF binding to monocytes and macrophages is incompletely characterized, although it appears to be mediated, at least in part, a member of the LRP/chylomicron remnant receptor (Misra *et al.* (1994) *J. Biol. Chem.* 269:18303-306).

## V. Pharmaceutical applications

### A. Indications

High dosage parenteral administration of lactoferrin or a pharmaceutical composition containing the same can be used in a variety of therapeutic and prophylactic applications. Furthermore, it will be clear that lactoferrin, and the fragments and variants thereof as described herein may be used in the preparation of medicaments, such as the

pharmaceutical compositions described herein, for the treatment and/or prophylaxis of the indications described herein.

For example, the methods are useful in treating various microbial infections such as bacterial infections. The methods are also useful in providing anti-inflammatory,  
5 anti-viral and anti-infective activities, as well a pro- and anti-coagulant effects, modulation of complement activation, inhibition of LPS mediated activation of neutrophils, regulation of transcription, growth promotion of intestinal epithelial cells, inhibition of hydroxyl-radical formation, and a role in intestinal iron uptake and excretion. Other properties and biological activities of LF are described in Nuijens *et al.*  
10 (1996) *J. Mammary Gland Biology and Neoplasia* 1:3, 283-293, which is incorporated herein by reference in its entirety and for all purposes.

hLF variants and neutralized LF have generally the same activities and uses as natural LF, except that deletion of the arginines of the first basic cluster results in reduction in binding to heparin, Lipid A, DNA, lysozyme, and cell surface sulfated  
15 molecules. Thus, some LF fragments can be administered to a patient to effect certain LF-mediated physiological changes (*e.g.*, regulation of cytokines) without causing other physiological consequences of LF administration (*e.g.*, neutralization of heparin by binding). Some lactoferrin fragments have various activities associated with the first cationic cluster of hLF. Such polypeptides are especially useful in selectively triggering  
20 responses involving the first cationic domain while avoiding the activation of responses associated with the binding of the second cationic domain and/or activities related to the iron binding activities of hLF. The neutralized hLF and hLF variants have a variety of advantageous properties. For example, hLF variants lacking the first basic cluster are particularly useful for initiating hLF-receptor-mediated immune and inflammatory  
25 responses (*e.g.*, reducing cytokine release, activation of natural killer cells, and anti-tumor effects), efficient receptor mediated delivery of nutritional iron, and other biological effects.

Some lactoferrin fragments can bind and neutralize heparin and LPS, lipid A, and DNA and hLZ. Some lactoferrin fragments can also bind to various target cells. LF binds  
30 to cell surfaces through two classes of LF binding sites: relatively low affinity sites which are cell surface sulfated molecules (*e.g.*, cell surface proteoglycans or glycosaminoglycans) and high affinity receptors. Since binding of LF to the low affinity sites involves the first cationic domain, some lactoferrin fragments can selectively bind to the low affinity sites without activating the LF high affinity receptors. Thus, for example,

some lactoferrin fragments are useful in neutralizing heparin or LPS without activating the LF high affinity receptor. Some lactoferrin fragments can neutralize the anticoagulant activity of heparin (including low molecular weight heparin). By neutralizing bacterial LPSs, some lactoferrin fragments can reduce the inflammatory response associated with these compounds. Such lactoferrin fragments are also used to inhibit viral entry into cells. Cell binding assays are well-known and are described in, *e.g.*, Mazurier (1989) *Eur. J. Biochem.* 179:481-87.

Cells with which lactoferrin, its fragments and variants can interact with include, intestinal cells (Hu *et al.* (1990) *Biochemistry* 29:535-541; Kawakam *et al.* (1991) *Am. J. Physiol.* 261:G841-G846; Mikogami *et al.* (1994) *Am. J. Physiol.* 267:G308-G31), mammary gland epithelial cells (Rochard *et al.* (1992) *Anticancer Res.* 1:2047-2052), hepatocytes (Regoecci *et al.* (1985) *Am. J. Physiol.* 248:G8-G14; MacAbee *et al.* (1991) *J. Biol. Chem.* 226:23624-23631; Ziere *et al.* (1992) *J. Biol. Chem.* 267:11229-11235), monocytes (Ismail *et al.* (1993) *J. Biol. Chem.* 268:21618-21625), activated lymphocytes (Mazurier *et al.* (1989) *Eur. J. Biochem.* 179, 481-487) and platelets (Leveugle *et al.* (1993) *Eur. J. Biochem.*, 213:1205-1211), each of which is incorporated by reference in their entirety for all purposes.

Intact lactoferrin and certain fragments and variants can also be used to inhibit entry into a cell of viruses, for example, cytomegalovirus (CMV), human immunodeficiency viruses (HIV) or herpes simplex virus 1 (HSV1) viruses. While not intending to be limited to this particular explanation, the antiviral action is thought to be mediated by interaction of hLF with cell surface proteoglycans (*e.g.*, heparin) employed by viral particles for cell entry, and/or by the stimulation of natural killer cells.

Therapeutic indications include use in therapy or prophylaxis of infection, including local infection, large scale (bacterial) infection, blood-borne infection (sepsis), as well as inflammation resulting from an infection or non-infectious inflammatory diseases (*e.g.*, chronic inflammatory disease of the ileum or colon). The compositions can also be used to prepare or treat organ transplant recipients or other immunosuppressed individuals (*e.g.*, AIDS patients) against the effects of infections.

The pharmaceutical compositions are effective in treating a variety of microbial infections, such as various viral and bacterial infections. For example, the compositions are effective in treating Gram-negative and Gram-positive bacteria. More specifically, some examples of pathogenic bacteria causing infections treatable by methods of the invention include: *Listeria*, *Escherichia*, *chlamydia*, *rickettsial* bacteria, *mycobacteria*,

*staphylococci, streptocci, pneumococci, meningococci and conococci, Klebsiella, proteus, serratia, pseudomonas, Legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lyme disease bacteria.*

Some examples of pathogenic viruses causing infections treatable by methods of the invention include: *hepatitis* (A, B, or C), *herpes virus* (e.g., VZV, HSV-I, HAV-6, HSV-II, and CMV, *Epstein Barr virus*), *adenovirus*, *influenza virus*, *flaviviruses*, *echovirus*, *rhinovirus*, *coxsackie virus*, *coronavirus*, respiratory syncytial virus (RSV), *mumps virus*, *rotavirus*, *measles virus*, *rubella virus*, *parvovirus*, *vaccinia virus*, HTLV virus, *dengue virus*, *papillomavirus*, *molluscum virus*, *poliovirus*, *rabies virus*, JC virus, *arboviral encephalitis virus*, and human immunodeficiency virus (HIV virus; e.g., type I and II).

Some examples of pathogenic fungi causing infections treatable by methods of the invention include: *Candida* (e.g., *albicans*, *krusei*, *glabrata*, *tropicalis*), *Cryptococcus neoformans*, *Aspergillus* (e.g., *fumigatus*, *niger*), Genus *Mucorales* (*Mucor*, *Absidia*, *Rhizopus*), *Sporothrix schenckii*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Coccidioides immitis* and *Histoplasma capsulatum*. Some examples of pathogenic parasites causing infections treatable by methods of the invention include: *Entamoeba histolytica*, *Balantidium coli*, *Naegleria*, *Fowleri*, *Acanthamoeba sp.*, *Giardia lamblia*, *Cryptosporidium sp.*, *Pneumocystis carinii*, *Plasmodium vivax*, *Babesia microti*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii* and *Plasmodium falciparis*.

Lactoferrin is particularly useful for the treatment of inflammatory diseases. This can occur, as noted above, through neutralization of bacterial LPSs (see also Lee et al., Infect. Immun 66, 1421-6 (1998), as well as through a reduction in cytokine production and neutrophil degranulation. Thus, in another aspect, the invention provides methods in which hLF or an LF variant is administered to a patient to reduce inflammation, for example in chronic inflammatory bowel disease (e.g., Crohn's disease and ulcerative colitis). Administration of hLF and hLF variants is useful for reducing reperfusion injury in a patient after myocardial infarction. hLF can be administered to neutralize bacterial LPS. The LPS binds through the first basic cluster, and is cleared from circulation via the second basic cluster.

The invention also provides methods in which lactoferrin is administered to a patient to inhibit myelopoieses and reduce production of GM-CSF.

Intact hLF, hLF variants and neutralized hLF are also useful for reducing or inhibiting release of a cytokine, such as IL-1, IL-2 or TNF-alpha, from LF-receptor

bearing cells in a patient, by administering LF or a LF variant. LF has been shown to reduce the release of cytokines, *e.g.*, IL-1, IL-2, and TNF-alpha from cells, and inhibit proliferation in mixed lymphocyte cultures (Chierici *et al.* (1994) *Acta Pediatr Suppl* 402:83-89). Suppression of IL-1 and TNF-alpha release from monocytes in response to LPS by hLF and variants is expected to down regulate recruitment and activation of neutrophils at inflammation sites (*see, e.g.*, Lonnerdal *et al.* (1995) *Ann Rev Nutr* 15:93-110). The suppressive effects of LF are thought to be mediated through the binding of LF to monocyte lactoferrin-receptors (Miyazawa *et al.* (1991) *J. Immunol.* 146:723-729), and can be responsible for the prophylactic effect of LF in mice injected intravenously with a lethal dose of *E. coli* (Sanchez *et al.* (1992) *Arch Dis Child.* 67:657-661) since LPS-mediated TNF responses in mice were attenuated by prior administration of LF (Lonnerdal *et al.*, *supra*). Methods for measuring cytokine release are well known (*e.g.*, ELISA). A reagent can be said to reduce or inhibit release of a cytokine from a cell when the level of cytokine release in the presence of the reagent is less than about 90%, more often less than about 70%, and most often less than about 50% of the levels released in the absence of the reagent under the conditions of the assay.

Intact lactoferrin, fragments and variants thereof can be administered to a patient to reduce TNF-alpha-mediated neutrophil degranulation. Neutrophils have been implicated as important mediators in both generalized and local inflammatory reactions, including sepsis, rheumatoid arthritis and ulcerative colitis. For example, clinical studies using anti-TNF monoclonal antibodies indicate that TNF, and likely the TNF-mediated activation of neutrophils, plays an important role in the pathophysiology of rheumatoid arthritis and ulcerative colitis.

Intact lactoferrin, fragments and variants thereof are useful for stimulating natural killer (NK) cells in the patient. Because hLF and LF variants cause stimulation of natural killer (NK) cells, the LF variants are useful for combating the targets of NK cells, *e.g.*, tumors, viruses and intracellular pathogens. Stimulation of natural killer (NK) cells by LF has been shown *in vitro* (Shau *et al.*, 1992, *J. Leukoc. Biol.* 51:343-349) and *in vivo* (Bezault *et al.*, 1994, *Cancer Res.* 54:2310-2312). NK cells can be stimulated in a patient by administering to the patient a composition comprising a hLF variant and a pharmaceutical excipient. hLF and LF variants can also be administered to a patient to inhibit growth of a solid tumor. A single intraperitoneal injection of LF inhibited growth of solid tumors induced by subcutaneous injection of syngeneic fibroblast-derived tumor

cell lines in mice (Bezault *et al.*, *supra*). LF variants are thus useful for stimulation of NK cells without neutralization of heparin or other undesirable effects.

In other methods, intact lactoferrin, fragments and variants thereof are used to deliver iron to an LF-receptor-bearing cell in a patient by administering to the patient a composition of hLF or a LF fragment or variant which is at least about 95% saturated with iron. Administration of these compounds are beneficial, for example, in treatment of anemia or iron storage diseases. LF- or LF-variant bound iron is delivered to a cell when the polypeptide-iron complex binds to a cell receptor and is internalized by the cell. Thus the compositions disclosed herein are suitable for use in baby formula as well as for administration to patients with disturbances in iron metabolism (*e.g.*, ferriprive anemia and iron storage diseases, and iron deficiency anemia of premature infants). LF variants can be saturated with iron following the procedure described in van Berkel *et al.*, 1995, *Biochem J.* 312, 107-114. A LF variant is at least 3% saturated with iron, more usually 80% saturated, most often at least 95% saturated and often more than 99% saturated. LF variants lacking the first basic cluster, or both the first and second basic clusters are particularly useful when the iron binding activities of LF are desired and when the activities mediated by basic clusters 1 and 2 (*e.g.*, heparin binding, high affinity receptor interaction) are not desired.

## B. Pharmaceutical Compositions

Intact lactoferrin, fragments and variants can be used as pharmaceutical, food additives, nutritional supplements, and the like. Such pharmaceutical compositions are usually administered parenterally, preferably intravenously. Intradermal, topical, enteral or intramuscular administration is also possible in some circumstances. Oral administration can also be used, optionally but not necessarily, in conjunction with parenteral administration.

The compositions containing the compounds can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications, particularly symptoms associated with a microbial infection, or otherwise prevent, hinder, retard, or reverse the progression of disease or infection or any other undesirable symptoms in any way whatsoever. An amount adequate to accomplish this is defined as "therapeutically effective amount or dose." Such effective dosage depends on the nature



and severity of the disease or condition, and on the general state of the patient's health, but is usually be between 10 and 100 mg of intact human lactoferrin per kilogram of body weight. In some methods, the dosage is at least 20, 30, 40, 50, or 60 mg/kg for intact lactoferrin. Fragments or variants having different molecular weights than intact  
5 lactoferrin can be administered in equimolar ratio to the above dosages or can be administered in higher dosages up to the mg/kg dosage given for intact lactoferrin. Dosages of 10, 20, 30, 40, 50, 60 and 100 mg/kg intact hLF are equal to 130, 260, 390, 520, 650, 780 and 1300 nmol/kg respectively (hLF Mr 77,000). Thus, for example, if a peptide/fragment has Mr 1500, a dosage of 0.19 mg/kg is in equimolar ratio to a dosage  
10 of 10 mg/kg intact human lactoferrin. The concentration of the polypeptide in the pharmaceutical composition can vary widely, *i.e.*, from less than about 0.1% by weight, usually being at least about 1% by weight, to as much as 20% by weight or more.

In prophylactic applications, intact lactoferrin, fragments or variants thereof, or pharmaceutical compositions containing the same are administered to a patient  
15 susceptible to or otherwise at risk of a particular disease or infection. Such an amount is defined to be a "prophylactically effective" amount or dose. In this use, the precise amounts again depends on the patient's state of health and weight. Typically, the dose is between 10 and 100 mg of intact human lactoferrin per kilogram of body weight. In some methods, the dosage of intact human lactoferrin is at least 20, 30, 40, 50, or 60 mg/kg.  
20 Dosages for fragments and variants can be adjusted to be in equimolar ratio with dosages specified for intact human lactoferrin as described above. Alternatively, fragments and variants can be administered at dosages up to the mg/kg dosage indicated for intact human lactoferrin.

In both therapeutic and prophylactic methods, lactoferrin, fragments and variants  
25 thereof can be administered as a single dosage or as a series of dosages over time. In some methods, a dosage of amount described above is administered at least daily for a period of a week, a month or a year. In other methods, a dosage of the above amount is administered at least twice weekly or weekly for a period of a month, a year, the duration of a disease or for the life of the patient.

30 Compositions prepared for intravenous administration typically contain 100 to 500 ml of sterile 0.9% NaCl or 5% glucose optionally supplemented with a 20% albumin solution and 100 to 500 mg of intact lactoferrin. Sterile Ringer's solution can also be used. A typical pharmaceutical composition for intramuscular injection would be made up to contain, for example, 1 ml of sterile buffered water and 10 to 100 mg of intact lactoferrin

Methods for preparing parenterally administrable compositions among others are well-known in the art and described in more detail in various sources, including, for example, *Remington's Pharmaceutical Science*, Mack Publishing, Philadelphia, PA, 17th ed., (1985) and Langer, *Science* 249:1527-1533 (1990) (both incorporated by reference in  
5 their entirety for all purposes).

The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the  
10 combination. Typically the hLF, or hLF polypeptide fragments or variants are administered along with a pharmaceutical excipient or carrier comprising any compatible, non-toxic substance suitable to deliver the polypeptides to the patient. Examples of such diluents are sterile water, distilled water, buffered water, physiological saline, PBS, Ringer's solution, dextrose solution, Hank's solution., alcohol, fats, waxes, and inert  
15 solids and can be used as the excipient or carrier. In addition, the pharmaceutical composition or formulation can also include other pharmaceutically acceptable carriers, adjuvants, or non-toxic, non-therapeutic, non-immunogenic stabilizers, excipients, buffering agents, dispersing agents, and the like. The compositions can also include additional substances to approximate physiological conditions, such as pH adjusting and  
20 buffering agents, toxicity adjusting agents, wetting agents, detergents and the like. Because of the ability of hLF to bind iron, in some instances it can be beneficial to include iron in the pharmaceutical composition. The concentration of the polypeptide in the pharmaceutical composition can vary widely, *i.e.*, from less than about 0.1% by weight, usually being at least about 1% by weight to as much as 20% by weight or more.

25 The composition can also include any of a variety of stabilizing agents, such as an antioxidant for example. Moreover, the polypeptides can be complexed with various well-known compounds that enhance the *in vivo* stability of the polypeptide, or otherwise enhance its pharmacological properties (*e.g.*, increase the half-life of the polypeptide, reduce its toxicity, enhance solubility or uptake). Examples of such modifications or  
30 complexing agents include the production of sulfate, gluconate, citrate, phosphate and the like. The polypeptides of the composition can also be complexed with molecules that enhance their *in vivo* attributes. A list of such molecules, provided by way of example and not limitation, includes carbohydrates, polyamines, amino acids, other peptides, ions (*e.g.*, sodium, potassium, calcium, magnesium, manganese), and lipids.

For oral administration, optionally in conjunction with parenteral administration, the active ingredient can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. The active component(s) can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that can be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, and edible white ink. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance. Pharmaceutical compositions can be administered with a foodstuff, typically milk, *e.g.*, bovine milk. This mode of administration have advantages when the LF/variant is produced by expression in a transgenic animal such as a transgenic bovine, goat, or rabbit. The production of LF in transgenic bovine milk is desirable since it provides a matrix wherein little or no purification is necessary for human consumption.

If desired, for example in the treatment of infections or disorders of the digestive tract or even for general oral administration of the compositions, it is possible to formulate solid or liquid formulations in an enteric-coated or otherwise protected form. In the case of liquid formulations, the formulation can be mixed or simply coadministered with a Prostatant, such as a liquid mixture of medium chain triglycerides, or the formulation can be filled into enteric capsules (*e.g.*, of soft or hard gelatin, which are themselves optionally additionally enteric coated). Alternatively, solid formulations comprising the polypeptide can be coated with enteric materials to form tablets. The thickness of enteric coating on tablets or capsules can be, for example, from 0.5 to 4 microns in thickness. The enteric coating can comprise any of the enteric materials conventionally utilized in orally administrable pharmaceutical formulations. Suitable enteric coating materials are known, for example, from *Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, 17th ed. (1985); and *Hagars*

*Handbuch der Pharmazeutischen Praxis*, Springer Verlag, 4<sup>th</sup> ed., Vol. 7a (1971), both of which are incorporated herein by reference in their entirety.

Another delivery option involves loading the composition into lipid-associated structures (*e.g.*, liposomes, or other lipidic complexes) which can enhance the pharmaceutical characteristics of the polypeptide component of the composition. The complex containing the composition can subsequently be targeted to specific target cells by the incorporation of appropriate targeting molecules (*e.g.*, specific antibodies or receptors). It is also possible to directly complex the polypeptide with a targeting agent.

The particular form of the composition varies with the intended mode of administration and therapeutic application. Typically, however, the composition includes polypeptides comprising full-length LF, fragments of LF, and/or variants of LF, and a pharmaceutically acceptable excipient. The polypeptide included in the composition has a defined length, can consist of one or more arginine residues at the N-terminus and have antimicrobial activity (*e.g.*, is effective in killing viruses or bacteria). In certain compositions, the polypeptide includes a contiguous segment of up to 27 amino acids and the N-terminal amino acids of the polypeptide consists of the residues XRR (where X is any amino acid and R is arginine. Some compositions include polypeptide no longer than 26 amino acids and the N-terminal amino acids are RR. Some compositions include a polypeptide no longer than 25 amino acids and the N-terminus is R (*i.e.*, Arg). In other instances, the polypeptide is shorter, such as 5, 10, 15, 20 or 25 amino acids long, or any length therebetween. The polypeptide is even smaller in other compositions. For instance, the polypeptide can simply consist of the N-terminal XRR or RR residues. The polypeptides used in the pharmaceutical compositions can also include any of the polypeptides described above.

While the N-terminus of the polypeptide consists of the amino acids XRR, RR or R, the remaining contiguous amino acid sequence of the polypeptide sequence can vary so long as the polypeptide has antimicrobial activity. For example, the remaining contiguous sequence can consist of a contiguous amino acid sequence from hLF, especially the sequence beginning after Arg<sup>3</sup>. Thus, the polypeptide can be hLF(1-11), hLF(2-11) or hLF(3-11). When the polypeptide used in the pharmaceutical composition consists of a contiguous amino acid sequence from the N-terminal segment of hLF, the amino acid sequence can include sequences wherein a small number (*e.g.*, one, two or three) amino acids are inserted or removed from the hLF sequence. Alternatively, the polypeptide includes contiguous sequences from hLF, wherein one or more of the amino acids has

been chemically modified.

Particularly when the compositions are to be used *in vivo*, the components used to formulate the pharmaceutical compositions are preferably of high purity and are substantially free of potentially harmful contaminants (*e.g.*, at least National Food (NF) grade, generally at least analytical grade, and more typically at least pharmaceutical grade. To the extent that a given compound must be synthesized prior to use, the resulting product is typically substantially free of any potentially toxic agents, particularly any endotoxins, which may be present during the synthesis or purification process.

Compositions for parental administration are also sterile, substantially isotonic and made under GMP conditions.

#### VI. Monitoring

In some methods, a patient is monitored after administration of lactoferrin, fragments or variants thereof to determine beneficial effects and/or side effects responsive to administration of lactoferrin. Monitoring can be for subjective symptoms, such as headache or nausea reported by the patient, physiological characteristics, such as temperature, presence of rash, and blood pressure, or by biochemical analysis of tissue samples from the patients. Side effects are deemed insubstantial if they do not prevent repeated administration of the same drug to the patient. Typically, insubstantial side effects resolve spontaneously within 24 hr, and preferably within 12 hr. Typically, insubstantial side effects do not recur with greater severity on repeated administration of the drug.

## EXAMPLES

### I. Introduction

During coronary artery bypass surgery (CABG) operations heparin in doses of 300-500 U/kg is used to prevent the blood from coagulating and to prevent thrombosis. After the patient's normal perfusion has been restored (i.e. after termination of the operation), the action of heparin needs to be terminated quickly to prevent hemorrhaging. The action of heparin can be terminated by recombinant rhLF, since rhLF binds to heparin and can prevent the anticoagulant effect of heparin.

This Example describes a clinical study in healthy male volunteers that examined the safety of four different dose levels of intravenous rhLF alone and / or in combination with heparin. In addition, the effect of rhLF on the neutralization of heparin and the pharmacokinetics of rhLF was investigated. The volunteers were also monitored for any signs of adverse reactions due to heparin or rhLF.

### II. Methods

#### Materials

The products administered as single intravenous doses were recombinant human lactoferrin; heparin; placebo recombinant human lactoferrin; placebo heparin. The study medication was manufactured from transgenic bovine milk under supervision of Pharming B.V., Leiden, The Netherlands, purified by CLB, Amsterdam, and filled and finished by Chiron, Amsterdam, The Netherlands. The sterile drug solution was packed in glass vials. All medication was stored at -20°C. Heparin was manufactured by Leo Pharmaceutical Products B.V. and was stored at the pharmacy department of Pharma Bio-Research Group B.V.

Human lactoferrin was purified from the milk of transgenic bovine using the following method. Raw milk containing 0.5 – 5.0 g human lactoferrin /l was pooled and processed in batches of 200 l. Raw milk was defatted by centrifugation and skimmed milk was stored at < -20°C. A thawed 200 l batch of hLF containing skimmed milk was incubated with 10% (w/v) S Sepharose (Pharmacia) for 12 ± 4 hours at 4°C. Beads were allowed to settle for at least 5 hours and the supernatant was removed. The Sepharose was then washed batchwise with 6 x 1 volume 20 mM Na-phosphate pH 7.5, 0.1 M NaCl, and 7 x with 1 volume 20 mM Na-phosphate pH 7.5, 0.4 M NaCl. A BPG 300 column

(Pharmacia) was packed with the S Sepharose containing the bound hLF and washed with 3 volumes of 20 mM Na-phosphate pH 7.5, 0.4 M NaCl, at 42 l/h. The column was eluted with 20 mM Na-phosphate pH 7.5, 1.0 M NaCl, at 42 l/h. The S Sepharose eluate was concentrated to 10 l and buffer exchanged to 20 mM Na-phosphate pH 7.5, 0.1 M NaCl using Hemoflow (Fresenius, 30kD cut-off). The preparation was filtrated (0.22 µm Millipore) and subjected to virus inactivation by adjusting to 1% (w/v) Tween 80 and 0.3% (w/v) tri-n-butyl phosphate and incubating for 15 ± 3 hours at 25 ± 1°C. The virus inactivated sample was loaded at 42 l/h onto a Q Sepharose column (Pharmacia), 180 x 80, serially connected to a Macroprep High S Support (MPHS, Biorad) 300 x 15. Both columns were equilibrated in 20 mM Na-phosphate pH 7.5, 0.1 M NaCl. The columns were washed with 10 litres of 20 mM Na-phosphate pH 7.5, 0.1 M NaCl at 42 l/h. The Q Sepharose column was disconnected and the MPHS column was washed with 40 volumes of 20 mM Na-phosphate pH 7.5, at 126 l/h. The MPHS column was stepwise eluted with first 8 volumes of 20 mM Na-phosphate pH 7.5, 0.4 M NaCl, at 42 l/h, and then 3 volumes of 20 mM Na-phosphate pH 7.5, 0.5 M NaCl, at 42 l/h. Fractions were tested for human lactoferrin content and for bovine lactoferrin impurity. Human lactoferrin containing fractions free of bovine lactoferrin were pooled and concentrated to 10 litres with Hemoflow (Fresenius, 30 kD cut-off). The concentrate was filtrated over 2 serially placed Planova 15 N filters (Asahi, 15 nm, 1 m<sup>2</sup>) for virus removal using a pressure of 0.5 bar. Again using the Hemoflow (Fresenius, 30 kD cut-off) the hLF preparation was concentrated to 9% and buffer exchanged to saline (0.9% NaCl). Finally the hLF preparation was sterile filtrated (Millipore, 0.1 µm) and stored at < -65°C.

#### Overall Study Design and Plan

The trial was a double blind, randomized, parallel group, controlled, ascending single intravenous dose, single center study and was conducted in 31 healthy male volunteers (Group I: n=4; Group II-IV: n=9). Subjects were randomized into group I (rhLF) or into one of three groups on a 4:4:1 basis (4 heparin + rhLF, 4 heparin placebo + rhLF, heparin + rhLF placebo).

#### Treatments Administered

In Group I four subjects received 2.5 mg/kg rhLF. In Groups II-IV subjects were randomized to either the previous rhLF dose following heparin infusion, the next rhLF

dose following placebo heparin or to heparin followed by placebo rhLF. For an overview of the groups, subject numbers and medication see Table 9.4.1-1.

Table 9.4.1-1 Treatment schedule

Group	Subjects	Treatment
I	01-04	2.5 mg/kg rhLF
IIa	08, 10, 12, 13	Heparin + 2.5 mg/kg rhLF
IIb	05, 06, 07, 11	Placebo heparin + 10 mg/kg rhLF
IIc	09	Heparin + placebo rhLF
IIIa	14, 16, 17, 20	Heparin + 10 mg/kg rhLF
IIIb	15, 18, 19, 22	Placebo heparin + 30 mg/kg rhLF
IIIc	21	Heparin + placebo rhLF
IVa	23, 25, 28, 29	Heparin + 30 mg/kg rhLF
IVb	26, 27, 30, 31	Placebo heparin + 60 mg/kg rhLF
IVc	24	Heparin + placebo rhLF

5

Subjects of Group I received on day 1 at t=0 a single dose of 2.5 mg/kg rhLF over a period of 10 minutes. Subjects of Groups II-IV received on day 1 at t=-15 minutes a single dose of heparin (15000 Units) or heparin placebo intravenously over a period of 5 minutes. At t=0 subjects received a single dose of rhLF or placebo-rhLF administered intravenously over a period of 10 minutes.. After a preceding dose was demonstrated to be safe, the next group received the following single dose.

#### Pharmacodynamic and pharmacokinetic assessments

To examine the efficacy of rhLF to neutralize the anticoagulation effect of heparin, the, pharmacodynamic parameters, including APTT, platelet aggregation, transferrin and total iron in blood samples, were reported. These parameters were used for evaluation of efficacy of rhLF administration to neutralizing the anticoagulant action of heparin.

Pharmacokinetic parameters determined from plasma concentration-time data for rhLF were:

$C_{max}$  maximum plasma concentration;



$t_{\max}$  time attain first maximum plasma concentration;

$t_{1/2}$  terminal elimination half-life;

$AUC_{0-t}$  area under the plasma concentration-time curve up to time  $t_{\text{last}}$ , where  $t_{\text{last}}$  is the last time point with a concentration above the lower limit of quantitation (log-linear trapezoidal rule);

$\hat{AUC}_{0-\infty}$  total AUC after extrapolation from time  $x$  to time infinity,  $(AUC_{0-t} + c/\lambda_z)$ , where  $c$  is the approximated concentration at  $t_{\text{last}}$  using the regression results of  $\lambda_z$ .

Analysis of the plasma samples was performed using a validated enzyme-linked immunosorbent assay (ELISA) method in the concentration and a Western Blot method.

For APTT a 3 ml blood sample was collected in a Na-citrate tube. Blood was kept on ice until centrifugation at 3000 rpm for 10 minutes at 4°C. For platelet aggregation a 10 ml blood sample was collected in a Na-citrate tube. Blood samples were taken from a non-compressed arm, blood was not allowed to be kept on ice during handling. Samples were centrifuged at 18°C for 10 minutes at 1200 rpm. Following centrifugation, platelet-rich plasma was removed. The remaining of the sample was centrifuged at 4000 rpm for 10 minutes at 18°C, subsequently platelet-poor plasma was removed.

For assessment of transferrin and total iron, 2.5 ml blood was collected. Blood was centrifuged at 4°C for 10 minutes at 3000 rpm.

Blood samples were collected for the analysis of rhLF plasma concentrations. Blood samples ( 3 ml each) were collected at regular intervals on day1 at 0, 5, 10, 20, 30, 60, 90, 120, minutes, 4, 8, 24 and 48 h. Blood samples were collected into tubes containing EDTA (final concentration of 10 mM) and within 10 minutes centrifuged at 3000 rpm for 20 minutes at 4°C. Samples were stored at -70°C until analysis. The bioanalysis was performed by Pharma Bio-Research Group BV, Assen, The Netherlands.

#### Post-study screening

On day 7 and 22 subjects returned to the clinical research unit for post-study screening comprising: physical examination; vital signs, ECG; APTT assessment; platelet aggregation and bleeding time; safety biochemistry, hematology and urinalysis; blood sampling for anti body measurement against hLF and bLF; adverse events and concomitant medication recording.

### Safety assessments

Adverse events, vital signs, ECG-recordings, physical examination, clinical laboratory parameters, FEV<sub>1</sub>, Ivy-bleeding time, and antibody response against human lactoferrin (hLF) and bovine lactoferrin (bLF) were recorded as safety assessments.

5

### III- Results

Heparin followed by placebo rhLF, 2.5 or 10 mg/kg rhLF resulted in prolonged APTT values above the ULQ (249 seconds) from t=0 until t=4 h post medication. After t=4 h, the median APTT values decreased and returned to baseline at t=8 h post medication. The heparin-induced increase in APTT is diminished by 30 mg/kg rhLF. Heparin followed by 30 mg/kg rhLF resulted in prolonged APTT values above ULQ at t = 0 h, decreased immediately to about 75 seconds after rhLF administration, and were back to baseline at t = 8 h post medication. Administration of rhLF after placebo-heparin treatment did not affect APTT values throughout the study period.

15 Heparin administration seemed not to have an effect on the platelet aggregation. Median day -1 to t=0 values showed a decrease of about 15% in the four groups without heparin administration and a decrease of about 3-10% in the heparin treated groups before rhLF administration. Considerable variation in median values was observed during 4h after administration of rhLF or placebo-rhLF. No trends could be observed. Administration of rhLF after placebo-heparin seemed not to have an effect on platelet aggregation. Descriptive statistics are presented in Section 14.2.1-4.

20 Heparin administration did not affect the concentrations of transferrin and total iron. However, groups treated with rhLF (2.5, 10 and 30 mg/kg) and heparin showed higher total iron values as compared to the group treated with heparin alone, although this elevation is in the range of individual differences. Administration of rhLF did not affect transferrin concentrations. Administration of rhLF after placebo-heparin did neither affect transferrin nor total iron throughout the study period. Descriptive statistics are presented in Section 14.2.1-5 and 14.2.1-6.

25 In summary, heparin-induced increments (>10 times baseline values) in blood coagulation time, as measured by APTT, in healthy volunteers could shortly and partially be returned to levels of 2.5 times baseline values after administration of rhLF, but only at the dose of 30 mg/kg rhLF. This effect occurred at 10 minutes post dose. All other doses of rhLF, i.e. 2.5 and 10 mg/kg did not show any effect upon APTT values. In all four

30

treatment groups with heparin, the APTT values returned to baseline values at 8 h post dose. Other dynamic parameters such as platelet aggregation, plasma transferrin and total iron concentration, after heparin treatment, were not significantly affected by rhLF administration.

- 5        Considering the total number of subjects participating in this study, very few adverse events were observed. Three moderate intensity adverse events occurred, of which two were classified as being possibly related to medication. These included an allergic reaction and nausea. Ivy bleeding time was not significantly affected by heparin and / or rhLF treatment.

10

#### IV. Discussion

- The highest dose of recombinant human Lactoferrin (30 mg/kg) given in combination with heparin used in the present study was capable of partially inhibiting the effect of heparin on blood coagulation time within 10 minutes. Administration of 15000  
15    U heparin i.v. alone induced a sharp increase in APTT from baseline values (approximately 30 seconds) to values above the upper limit of quantitation (249 seconds). This lasted for 120 minutes after placebo, 2.5 or 10 mg/kg rhLF administration. RhLF administration at a dose of 30 mg/kg was able to partially block the effect of heparin on blood coagulation, resulting in a sharp decrease in APTT immediately after rhLF  
20    administration to approximately 2.5 times baseline values (approximately 75 seconds). However, after the initial blocking effect of rhLF on the heparin-induced increase in APTT, the APTT values increased again to approximately 100 seconds. The return to baseline values was only achieved at  $t=8$  h post medication. The increase in APTT at  $t=2$  h, following the initial sharp decrease after 30 mg/kg rhLF administration might be the  
25    result of clearance of rhLF from the circulation by metabolic mechanisms, whereas the subsequent decrease in APTT at  $t=4-8$  h is probably caused by the clearance of heparin from the circulation.

- The present study shows maximal heparin activity until  $t=2$  h post dose (i.e. 135 minutes post heparin administration). Animal studies revealed total clearance of 25 mg/kg  
30    rhLF from the blood within 3 hours, and a half-life time of 45 minutes (a comparison with pharmacokinetic data derived from the present study is required, but these data are not available at this moment).

      A complete reversal of the anti-coagulating action of heparin by rhLF can be achieved by administration of a higher single dose of rhLF or by administration of rhLF

as a maintenance dose. If rhLF forms irreversible complexes with heparin, it is preferable to increase the dose of a single rhLF bolus infusion. However, if rhLF forms reversible complexes with heparin, a maintenance dose of rhLF infusion is preferred.

5 Other dynamic parameters such as platelet aggregation and plasma iron concentration measured after heparin treatment, were not significantly affected by rhLF administration.

Only a limited number of subjects experienced any adverse events and even these were minor. First, in Group IVb (placebo-heparin in combination with 60 mg/kg rhLF), two subjects experienced adverse events. One subject suffered a short but moderate  
10 period of nausea, duration 62 minutes. This reaction was thought to be possibly related to medication. The same subject also experienced a temperature changed sensation, possibly related and a pharyngitis, not related to medication. Another subject complained of headache and vision abnormality, both possibly related to medication and a myalgia not related to medication. Second, in Group IIIb (placebo-heparin in combination with 30  
15 mg/kg rhLF) one subject experienced an allergic reaction. This event included urticaria, difficulty in breathing, conjunctivitis and Quinke's edema. This event was classified as being possibly related to medication. Other mild adverse events occurred throughout the duration of the study, but were mainly unrelated to medication

It can be concluded that the intravenous administration of rhLF alone and in combination  
20 with heparin is safe.

It will be apparent from the foregoing that the invention includes a variety of uses. These usages include the use of high dosages of lactoferrin for the manufacture of a medicament for parenteral administration to a patient for the treatment or prophylaxis of a disease, such as an infectious disease or inflammation. For the purposes of clarity and  
25 understanding, the invention has been described in these examples and the above disclosure in some detail. It will be apparent, however, that certain changes and modifications can be practiced within the scope of the appended claims. All publications and patent filings = cited in the present application are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually  
30 denoted.

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**CLAIMS**

1. Use of lactoferrin, or a fragment or variant thereof, in the preparation of  
5 a medicament for the treatment or prophylaxis of a disorder selected from the group  
consisting of an infectious disease, an inflammatory disease, and an excess of heparin.

2. A use according to any one of claims 1 or 2, wherein the medicament is  
suitable for parenteral administering a dosage of at least 130 nmol lactoferrin, or a  
10 fragment or variant thereof, per kg body weight.

3. A use according to any one of the preceeding claims, wherein the  
administering is performed by intravenous injection.

4. A use according to any one of the preceeding claims, wherein the  
15 dosage is 260-1300 nmol/kg.

5. A use according to any one of the preceeding claims, wherein lactoferrin  
is intact human lactoferrin.  
20

6. A use according to any one of claims 1 - 4, wherein the lactoferrin  
fragment is a fragment comprising amino acids 1-47 of SEQ ID No. 1.

7. A use according to claim 6, wherein the lactoferrin fragment is a  
25 fragment comprising at least 6 but no more than 27 contiguous amino acids from the N-  
terminal segment of human lactoferrin in SEQ ID NO:1, wherein the N-terminus of said  
polypeptide is residue 1 of SEQ ID NO:1.

8. A use according to any one of claims 1 - 4, wherein the lactoferrin  
30 fragment is a fragment comprising at least 7 contiguous amino acids of SEQ ID NO:1.

9. A use according to any one of claims 1 - 4, wherein the lactoferrin  
variant a variant that binds heparin with lower affinity than does natural lactoferrin.

10. A use according to claim 9, wherein the lactoferrin variant is hLF-2N, hLF-3N, hLF-4N, or hLF-5N.

5 11. A use according to any one of claims 1 - 4, wherein the lactoferrin variant is between about 3% and about 100% saturated with iron.

12. A use according to claim 11, wherein the lactoferrin variant is at least about 95% saturated with iron.

10 13. A method of treating a patient, comprising parenterally administering a dosage of lactoferrin or a fragment or variant thereof, of at least 130 nmol/kg body weight to the patient.

15 14. The method of claim 13, wherein the administering is performed by intravenous injection.

15 15. A method according to claims 13 or 14, further comprising administering a second dosage of lactoferrin or a fragment or variant thereof, orally to the patient.

20 16. A method according to any one of claims 13 - 15, wherein the lactoferrin is intact human lactoferrin and the dosage is at least 30 mg/kg.

25 17. A method according to any one of claims 13 - 16, wherein the dosage is 260-1300 nmol/kg.

30 18. A method according to any one of claims 13 - 17, wherein the patient suffers or is susceptible to a disorder selected from the group consisting of an infectious disease, an inflammatory disease, and an excess of heparin and the dosage is sufficient to prevent, or treat the disorder.

19. A method according to any one of claims 13 - 18, wherein the patient is substantially free of side-effects response to administration of the lactoferrin.

20. A method according to any one of claims 13 - 19, wherein the dosage is administered daily for a period of at least a week.

21. A method according to any one of claims 13 - 20, wherein the  
5 lactoferrin is intact lactoferrin.

22. A method according to any one of claims 13 - 21, wherein the lactoferrin is intact human lactoferrin.

10 23. A method according to any one of claims 13 - 19, wherein the lactoferrin fragment is a fragment comprising amino acids 1-47 of SEQ ID No. 1.

24. A method according to claim 23, wherein the lactoferrin fragment is a fragment comprising at least 6 but no more than 27 contiguous amino acids from the N-  
15 terminal segment of human lactoferrin protein in SEQ ID NO:1, wherein the N-terminus of said polypeptide is residue 1 of SEQ ID NO:1.

25. A method according to any one of claims 13 - 19, wherein the lactoferrin fragment is a fragment comprising at least 7 contiguous amino acids of SEQ  
20 ID No:1.

26. A method according to any one of claims 13 - 19, wherein the lactoferrin fragment is a fragment comprising at least 6 but no more than 24 contiguous amino acids.  
25

27. A method according to any one of claims 13 - 19, wherein the lactoferrin variant is a variant that binds heparin with lower affinity than does natural lactoferrin.

30 28. A method according to claim 27, wherein the lactoferrin variant is hLF-2N, hLF-3N, hLF-4N, or hLF-5N.

29. A method according to any one of claims 13 - 19, wherein the lactoferrin variant is between about 3% and about 100% saturated with iron.

30. A method according to claim 29, wherein the lactoferrin variant is at least about 95% saturated with iron.

## SEQUENCE LISTING

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 40 Ile Tyr Glu Ala Gly Leu Ala Pro Tyr Lys Leu Arg Pro Val Ala Ala  
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(54) **Method for heat treatment of lactoferrin without losing physiological activities thereof.**

(57) **A method for heat treatment of lactoferrin or a matter containing lactoferrin in moisturized state or a matter containing moisturized lactoferrin, comprising adjusting the pH of said moisturized lactoferrin between 1.0 - 6.5 both inclusive, heating said matter at a temperature equal to or higher than 60 °C for a span of time which may assure 60% or more of undenaturation rate of lactoferrin.**

**EP 0 437 958 A1**

# METHOD FOR HEAT TREATMENT OF LACTOFERRIN WITHOUT LOSING PHYSIOLOGICAL ACTIVITIES THEREOF

## FIELD OF THE INVENTION

The present invention relates to a method for heat treatment of lactoferrin or matters containing lactoferrin without losing physiological activities thereof. More particularly, the present invention relates to a method for heat treatment of lactoferrin or matters containing lactoferrin under a pH condition between 1.0 - 6.5 at a temperature over 60 °C.

## BACKGROUND OF THE INVENTION

Lactoferrin is known as an iron-binding protein distributed in tear, saliva, peripheral blood, milk and the like. It has been known that lactoferrin has various physiological activities, for example, antibacterial activity against harmful bacteria (B. J. Nonnecke, and K. L. Smith; Journal of Dairy Science; Vol. 67, p.3; 1984), activity for promoting iron absorption at the intestine (G. B. Fransson et al; Journal of Pediatric Gastroenterology and Nutrition; Vol. 2, p.693; 1983), anti-inflammatory activity (J. V. Bannister et al; Biochimica et Biophysica Acta; Vol. 715, p.116; 1982) and so on.

Therefore addition of lactoferrin to foods, processed foods, medicines, cosmetics and the like is desirable.

However, lactoferrin is unstable to heating at near neutral pH, and heat treatment of lactoferrin may result in denaturation of lactoferrin. It is reported that the physiological activities of lactoferrin are almost lost by heating at 62.5 °C for 30 minutes, and complete denaturation is occurred by heating at 70 °C for 15 minutes (J. E. Ford et al; Journal of Pediatrics, Vol. 90, page 29; 1977). In this connection, a typical condition of traditional thermal sterilization of milk is heating at 63 °C for 30 minutes.

It is often necessary to heat foods, feeds, medicines, cosmetics for pasteurization, sterilization, or cooking, however, sufficient thermal treatment could not be applied to lactoferrin or matters containing lactoferrin as an ingredient for utilizing its physiological activities.

The inventors of the present invention have exerted their efforts to develop a method for heat treatment of lactoferrin or matters containing lactoferrin without losing its physiological activities, and found that when lactoferrin or matters containing lactoferrin are heated under acidic conditions, its physiological activities such as antibacterial activity, iron-binding activity, antigenicity are scarcely affected. This invention based on this discovery.

## OBJECTS OF THE INVENTION

Therefore, it is an object of the present invention to provide a method for heat treatment of lactoferrin or matters containing lactoferrin without losing physiological activities thereof.

It is a particular object of the present invention to provide a method for pasteurization, sterilization or cooking by heating of lactoferrin or matters containing lactoferrin without losing physiological activities thereof.

## SUMMARY OF THE INVENTION

In accordance with the present invention, lactoferrin or matters containing lactoferrin are heated at a temperature over 60 °C under a pH condition between 1.0 - 6.5.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing relationships between heating temperature and heating time with respect to lactoferrin samples having different pH for assuring undenaturation rates

## DETAILED DESCRIPTION OF THE INVENTION

The word "lactoferrin" used herein includes lactoferrin derived from any and all sources of lactoferrin such as mammalian milk (for example, human milk as well as cow's, sheep's, goat's, horse's milk and the like) at any lactation stage (for example, colostrum, transitional milk, matured milk, milk in later lactation),

processed milk and byproducts in milk-processing such as skim milk, whey and the like (hereinafter they are referred to in general as milk and the like).

The word "lactoferrin" used herein include any and all lactoferrin substances such as commercial lactoferrin; native lactoferrin just isolated by the conventional method (for example, ion-exchange chromatography) from any and all sources of lactoferrin; apolactoferrin obtained by removing iron from native lactoferrin with hydrochloric acid, citric acid and the like; metal saturated lactoferrin obtained by chelating apolactoferrin with metal such as iron, copper, zinc, manganese and the like; or suitable mixtures thereof (hereinafter they are abbreviated as LF in general).

The words "matters containing lactoferrin" used herein include any and all matters which inherently contain LF, and any and all matters to which LF is added. For example, matters containing lactoferrin may include foods, feeds, cosmetics and medicines and the like which include LF. For the sake of simplicity, lactoferrin and matters containing lactoferrin will be referred to hereinafter as LF-matters in general.

Typically, heat treatment of LF-matters is performed in liquid phase, however, LF-matters are not necessarily in liquid phase, but the matters must contain lactoferrin in a state or a phase wherein LF is placed under a pH condition within the described range. For example, LF-matters may include coarse solid matters to which LF is mixed or stuck in a form of liquid, slurry or paste.

In accordance with the method of the present invention, LF-matters are first adjusted their pH within 1.0 - 6.5, preferably 2.0 - 6.0 by adding inorganic acid (for example, hydrochloric acid, phosphoric acid and the like) and/or organic acid (for example, acetic acid, citric acid and the like). Certainly, when LF-matters per se have a pH value within the described range, pH adjustment is unnecessary, however, it is still preferable to adjust the pH of LF-matters to the optimum pH depending upon the heating condition to be adapted (heating temperature and heating time) and the purpose of heating (for example, sterilization, cooking and the like).

The pH range was determined as the results of Test 1 which shows that LF solution tend to coagulate by heating under the pH range between 6.5 - 10.0 and tend to be hydrolyzed by heating under a pH value over 10.0 or below 1.0, however, denaturation or hydrolysis of LF can be minimized when it is heated at a temperature higher than 60 °C under the pH range between 1.0 - 6.5 for a relatively short time.

In accordance with the present invention, heat treatment is made at a temperature over 60 °C. The temperature range of heat treatment is mainly intended to sterilization, but it was determined with taking practical heat processing in food industry in consideration. Heating of LF-matters may results more or less in denaturation of LF and may affect physiological activities thereof. Potency of physiological activities of LF generally depend upon concentration of LF. Thus, in order to utilize physiological activities of LF, it is desirable to include undenaturated LF as much as possible in the heated LF-matters. In the practical point of view, undenaturation rate of LF in LF-matters is set as more than 60 % in the present invention, though it is not critical. The words "undenaturation rate" used herein means the rate of the quantity of undenaturated LF in the LF-matters after heating to that before heating.

In conclusion, heat treatment of LF-matters is performed under pH 1.0 - 6.5 at a proper combination of a temperature over 60 °C and a proper span of time to achieve an undenaturation rate over 60 %.

LF-matters treated by heating are cooled by the conventional method, if necessary, filled in containers, sealed, to thereby obtain LF products having physiological activities. LF-matters treated by heating can be dried to obtain powdery products. In this connection, any conventional drying methods can be adopted as far as LF is not further denaturated. Typical drying methods are vacuum drying, freeze drying and spray drying and the like.

It will be understood that the resultant LF-matters treated by heating can be used as it is as foods, feeds, cosmetics, medicines and the like depending upon other ingredients contained therein and can be added to fresh or processed foods or feeds, or materials thereof as well as medicines or cosmetics or materials thereof.

Now some exemplifying tests will be described for better understanding of the present invention.

#### [TEST 1]

The purpose of this test is to exemplify the relationships between the conditions of heat treatment and the degree of affection to physiological activities of LF.

#### 1) METHOD

Commercial LF (by Oleofina, Belgium) was dissolved into purified water in 5 % concentration (by weight, the same will be applied unless specifically noted), the resultant solution was distributed by 10 ml

into 66 test tubes. The LF solution in test tubes were adjusted to pH 1 - 11 as shown in Table 1 to prepare samples of 11 groups each consisting of 6 samples having the same pH value. Six samples belonging to each group were heated at 6 different temperatures, 60 °C - 120 °C for 5 minutes as shown in Table 1.

After heating, appearance of the samples were observed by naked eyes to find coagulation of LF, then degree of denaturation of LF in each sample was measured by reversed-phase high performance liquid chromatography using Asahi-Pack C4P-50 (trademark, by Asahi Kasei) with linear gradient of acetonitrile containing 0.5 M sodium chloride.

## 2) RESULTS

The results are shown in Table 1. The values in Table 1 denote undenaturation rates, i. e. the value of 100 % means that LF is not denaturated. It will be understood that LF is very stable to heating under acidic conditions. Also it will be noted that there are remarkable gaps between undenaturation rates of samples of pH 6 and 7 heated at 80 °C, those of samples of pH 6 and 7 heated at 90 °C, and those of samples of pH 5 and 6 heated at 100 °C. In order to interpolate the values between the gaps and to find preferable conditions of heat treatment under practical temperatures, similar test was carried out on the samples having smaller difference of pH values. More particularly, samples of pH 6.5 were heated at 80 °C and 90 °C, and a sample of pH 5.5 was heated at 100 °C for 5 minutes respectively. As the results, undenaturation rates thereof were 67.3 % (sample of pH 6.5 heated at 80 °C), 45.0 % (sample of pH 6.5 heated at 90 °C) and 63.4 % (sample of pH 5.5 heated at 100 °C) respectively.

It was confirmed that the preferable combinations of heating temperature and pH value at which an undenaturation rate over 60 % was resulted were: at 60 °C under pH 8 or less, at 70 °C under pH 7 or less, at 80 °C under pH 6.5 or less, at 90 °C under pH 6.0 or less, and at 100 °C under pH 5.5 or less.

Table 1

Temp. (°C)	Test Item	pH of LF Solution										
		1	2	3	4	5	6	7	8	9	10	11
60	coagul.	-	-	-	-	-	-	-	-	±	±	-
	undenatur.	91.2	100	100	100	100	100	100	70.4	35.5	19.7	9.8
70	coagul.	-	-	-	-	-	-	-	±	±	±	-
	undenatur.	87.5	100	100	100	100	100	100	51.3	24.1	10.0	0
80	coagul.	-	-	-	-	-	-	-	±	+	±	-
	undenatur.	71.1	93.5	100	100	100	100	26.8	12.1	x	0	0
90	coagul.	-	-	-	-	-	-	±	+	+	±	-
	undenatur.	50.7	80.4	93.5	100	100	95.7	15.2	x	x	0	0
100	coagul.	-	-	-	-	-	±	+	+	+	+	-
	undenatur.	14.4	25.7	45.6	100	95.8	5.3	x	x	x	x	0
120	coagul.	-	-	-	-	-	+	+	+	+	+	-
	undenatur.	0	0	0	0	0	x	x	x	x	x	0

Note: observation by naked eye:

+: completely coagulated ±: not coagulated but semitransparent

-: not coagulated and transparent

undenaturation rate by liquid chromatography:

numerical values: percentage of peak of heated LF to peak of unheated LF in chromatogram

x: unmeasurable due to coagulation

[Test 2]

The purpose of this test is to determine proper combinations of heating temperature and heating time under different pH values for achieving an undenaturation rate over 60 %.

#### 1) METHOD

Nine groups of samples of LF solutions having different pH values, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, and 8.0 were prepared in the same manner as in Test 1. The resultant solutions were heated at a temperature between 60 °C - 120 °C for 100 minutes. Undenaturation rate was measured for each of the samples in the same manner as in Test 1, using a portion thereof which was periodically took out and cooled. Intervals for taking out respective portions of samples were 30 seconds during 1 - 10 minutes after initiation of heating, 1 minute during 10 - 20 minutes, and 10 minutes during 20 - 100 minutes after initiation of heating.

## 2) RESULTS

The results are shown in Fig. 1 wherein combinations of heating temperature and heating time with which 60 % of undenaturation rate were resulted were plotted with respect to each of the samples of pH 1.0 represented by the line x - x, of pH 2.0 represented by  $\Delta$  -  $\Delta$ , of pH 3.0 represented by  $\square$  -  $\square$ , of pH 4.0 represented by  $\bigcirc$  -  $\bigcirc$ , of pH 5.0 represented by  $\bullet$  -  $\bullet$ , of pH 6.0 represented by  $\blacktriangle$  -  $\blacktriangle$ , of pH 6.5 represented by  $\odot$  -  $\odot$ , of pH 7.0 represented by  $\blacksquare$  -  $\blacksquare$ , and of pH 8.0 represented by  $\blacklozenge$  -  $\blacklozenge$ , and wherein the ordinate shows heating time as shown in logarithmic graduation and the abscissa shows heating temperature.

Any combinations of temperature and time in the regions below the respective lines in Fig. 1, may result in undenaturation rate over 60 % for respective LF-matters having the corresponding pH.

It was confirmed that LF-matters are stable under acidic pH condition between 1.0 - 6.5, especially between 2.0 - 6.0 and that even if LF-matters are heated at a relatively higher temperature, denaturation of LF can be kept at relatively low degree, if the heating time is shortened. For example, when an LF-matter of pH 4.0 is heated at 120 °C, undenaturation rate over 60 % can be resulted by restricting the heating time for 3 minutes or less, though the undenaturation rate was 0 % when the LF-matter is heated at 120 °C for 5 minutes (cf. Table 1). It will be understood that when an LF-matter of pH 2.0 is heated at 100 °C for 5 minutes, the undenaturation rate was lower than 60 % (25.7 % in Table 1), however, undenaturation rate over 60 % can be resulted if heating time is less than 2 minutes (cf. Fig. 1).

It will be understood that in order to achieve an undenaturation rate over 60 %, any combination of a heating temperature, a heating time and a pH value can be selected in the region below the line corresponding to the adopted pH value of an LF-matter taking the purpose of heat treatment in consideration.

### [TEST 3]

The purpose of this test is to determine the affection of heat treatment of LF to antibacterial activity.

#### 1) METHOD

##### 1-1) PREPARATION OF CULTURE MEDIUM AND PRE-CULTURE

###### 1-1-1) PREPARATION OF PRE-CULTURE

From the stock culture of Escherichia coli, bacterial cells was taken out with a platinum loop and spread onto a plate count agar (by Nissui Seiyaku), followed by stationary incubation at 35 °C for 16 hours (under aerobic condition). The colonies grown on the surface of the culture were collected with a platinum loop and suspended into pasteurized physiological saline solution to prepare pre-culture having optical density of 1.0 (at 660 nm) measured by a spectrophotometer (by Hitachi Seisakusho).

###### 1-1-2) PREPARATION OF BASIC CULTURE MEDIUM

Basic culture medium (liquid culture medium) was prepared with dissolving bactocasitone (by Difco) into purified water in 1 % concentration, adjusting the pH of the resultant solution to 7.0 by 1 M sodium hydroxide, then pasteurized at 115 °C for 15 minutes.

###### 1-1-3) PREPARATION OF TEST CULTURE MEDIUM

LF solution adjusted to pH 4.0 was heated (for pasteurization) at 100 °C for 5 minutes in the same manner as in Test 1 to prepare a test sample of heated LF solution. A quantity of the resultant heated LF



solution was added to a portion of the basic culture medium to prepare a test culture medium containing 1,000 ppm of heated LF.

LF solution adjusted to pH 4.0 was filtrated with a membrane filter (by Advantec) to remove any microbial cells contaminated therein to prepare unheated LF solution. A quantity of the resultant unheated LF solution was added to a portion of the basic culture medium to prepare a test culture medium containing 1,000 ppm of unheated LF.

#### 1-1-4) PREPARATION OF CONTROL CULTURE MEDIUM

Sterilized water of the same quantity to that of LF solutions for preparing test culture media was added to a portion of the basic culture medium to prepare a control culture medium containing no LF.

#### 1-2) TEST FOR ANTIBACTERIAL ACTIVITY

To each of the test and control culture medium, the pre-culture was inoculated in 1 % concentration, followed by incubation at 35 °C for 15 hours. The growth inhibition rate was determined by periodically measuring optical density of the culture broths after 5, 10 and 15 hours from the initiation of incubation in the same manner as previously described and calculated in accordance with the following formula.

growth inhibition rate (%) =  $100 - (A / B \times 100)$  (wherein A denotes the difference of the respective optical densities of the test culture medium after 5, 10 and 15 hours incubation and that of before incubation, B denotes the difference of the respective optical densities of the control culture medium after 5, 10 and 15 hours incubation and that of before incubation respectively)

In preparation of test and control culture medium, there was no substantial changes in pH values before and after addition of LF solutions or sterilized water to the basic culture medium.

#### 2) RESULTS

The results are shown in Table 2. It was confirmed that antibacterial activity of heated LF and unheated LF was almost the same.

Table 2

Sample	Proliferation Inhibition Rate (%)		
	after 5 hrs	after 10 hrs	after 15 hrs
Control	0	0	0
unheated LF	28.3	7.0	1.0
heated LF	23.3	9.9	1.2

#### [TEST 4]

The purpose of this test is to determine the affection of heat treatment of LF to iron binding property.

#### 1) METHOD

Heated LF solution (pH 4.0, heated at 100 °C for 5 minutes) and unheated LF solution were prepared in the same manner as in Test 1. Iron binding property of the resulted LF solutions was measured by the method of Baer et al (A. Baer et al, Journal of Dairy Research, Vol. 46, page 83, 1979) , and the percentage of the quantity of iron bound to heated LF to the quantity of iron bound to unheated LF was calculated.

#### 2) RESULTS

The results are shown in Table 3. Iron binding property of heated LF and unheated LF is almost the

same, and found that there is no substantial effect to iron binding property of LF by heat treatment.

Table 3

Sample	Iron Binding Property
unheated LF	100 %
heated LF	97 %

## [Test 5]

The purpose of this test is to determine the affection of antigenicity of LF by heat treatment.

## 1) METHOD

Antigenicity of samples of heated LF and unheated LF prepared in the same manner as in Test 4 (pH 4.0, heated at 100 °C for 5 minutes) were measured by Laurell's method (C. B. Laurell, Analytical Biochemistry, Vol. 15, page 45, 1966) , and the percentage of the value of antigenicity of heated LF to that of unheated LF was calculated.

## 2) RESULTS

The results are shown in Table 4. It was confirmed that antigenicity of heated LF is almost the same with that of unheated LF, and there is no substantial difference therebetween.

Table 4

Sample	Antigenicity (%)
unheated LF	100
heated LF	99

## Test 6

The purpose of this test is to exemplify the antibacterial activity of heated LF when it was added to milk.

To 10 kg of sterilized milk (sterilized at 70 °C for 30 minutes then cooled), 500 g of heated LF solution prepared in the same manner as in Example 3 (conditions of heat treatment: pH 5.0, at 70 °C, for 30 minutes) was aseptically added to prepare a test sample (Sample No. 1) . The resultant test sample was distributed into bottles by 200 ml and sealed. A control sample (Sample No. 2) which consisting of sterilized milk to which no LF was added (conditions of sterilization is the same with that of the milk used in preparation of test sample No. 1) and a control sample (Sample No. 3) which consists of sterilized milk (conditions of sterilization is the same with that of the milk used in preparation of test sample No. 1) and sterilized LF (deactivated) in the milk (concentration of LF is the same with that in Sample No. 1) were prepared.

The resulted samples were preserved at 25 °C to observe any change in appearance with naked eyes to determine the activity of the added LF.

All of the Sample Nos. 2 and 3 were coagulated by the 2nd day from the initiation of preservation, whereas there was observed no coagulation in Sample No. 1 by the 5th day from the initiation of preservation.

It will be understood that coagulation of milk means acidification or fermentation of milk. From the

results, it was confirmed that antibacterial activity of LF heated in accordance with the present invention is effective for preservation of milk. The results of this test make it possible to assume that LF heated in accordance with the present invention is effective also for preservation of any and all matters other than milk when the heated LF is added in the same manner thereto. It is also apparent that other physiological activities of the heated LF exemplified by the previous tests are maintained in the test sample (Sample No. 1).

Now, some examples will be described for better understanding of the present invention.

#### EXAMPLE 1

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To 29.7 kg of purified water, 300 g of commercial LF (by Oleofina, Belgium) was dissolved, then the pH of the resulted solution was adjusted to 4.0 with 1 M hydrochloric acid. The resultant solution was preheated at 70 °C for 3 minutes, then sterilized at 130 °C for 2 seconds using UHT sterilization system (by Morinaga Engineering), followed by cooling to 15 °C, thereby about 30 kg of heated LF solution was obtained.

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The undenaturation rate of the resultant LF solution was 99 % as measured by the same method as in Test 1 (liquid chromatography). There was no scorching and no sticking of LF to sterilization system during the heat treatment. The resultant LF solution can be used as it is as a medicine and can be used as an ingredient of pharmaceutical preparations or cosmetics and can be used as an additives for foods or feeds.

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#### EXAMPLE 2

To 20 kg of orange juice having the ingredient as shown in Table 5, 1 kg of commercial LF (by Oleofina, Belgium) was dissolved. The resultant solution was heated at 80 °C for 15 minutes, followed by cooling, aseptically distributing into glass bottles by 200 ml, sealed, thereby 95 bottles of orange juice were produced. The undenaturation rate of the heated LF in the orange juice was 99.6 % as measured by the same method as in Test 1 (liquid chromatography).

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Table 5

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sugar	5.0 %
perfume (orange)	0.2 %
citric acid	0.1 %
sodium citrate	0.05 %
<u>carbonated water</u>	<u>94.65 %</u>
Total	100

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Notes:

nature of the mixture: pH 4.5, liquid, transparent and orange in color

#### EXAMPLE 3

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To 9.5 kg of purified water, 500 g of commercial LF (by Oleofina, Belgium) was dissolved. The resulted solution was adjusted to pH 5.0 with 1 M citric acid. The resultant solution was heated at 70 °C for 30 minutes, followed by cooling, thereby about 10 kg of heated LF solution was obtained.

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#### EXAMPLE 4

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## A mixture of

5	salad oil	6.8 kg
	vinegar	1.4 kg
	lemon juice	0.13 kg
	egg yolk	1.37 kg
10	table salt	0.06 kg
	sugar	0.14 kg
	mustard	0.10 kg
15	commercial LF	0.1 kg (by Oleofina, Belgium)

was sufficiently stirred, then resulted mixture was heated at 60 °C for 10 minutes under stirring, followed by cooling, thereby about 10 kg of mayonnaise (pH 4.0) containing heated LF was obtained.

## EXAMPLE 5

## A mixture of

25	water	2.9 kg
	commercial apple juice	0.75 kg
	powdery agar	0.05 kg
30	granulated sugar	2.05 kg
	commercial LF (by Oleofina, Belgium)	50 g

was homogeneously mixed, the resulted mixture (pH 5.0) was heated at 90 °C for 20 minutes, the resultant solution was poured into a flat pan, followed by cooling. Resultant gel was cut into 2 cm cubes, followed by drying on a mesh, sprinkled with granulated sugar, thereby 500 pieces of jelly containing heated LF were produced.

## EXAMPLE 6

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In a column having 10 cm diameter, 500 ml of ion-exchange resin, CM-Toyopearl 650C (trademark, by TOSOH) was filled, followed by passing 2 l of 10 % NaCl solution into the column, washing by water, thereby Na type ion-exchange column was prepared. To the resultant column, 60 l of goat cheese whey (pH 6.5) was passed at the flow rate of 4 l/h at 4 °C. The components adsorbed to the resin were eluted by the conventional procedures to thereby obtained about 5 l of the eluate. The resultant eluate was dialyzed against purified water, followed by ultrafiltration with an ultrafiltration module SEP- 1013 (by Asahi Kasei) to concentrate the eluate, to thereby obtain about 200 ml of 1 % goat LF solution.

The resultant LF solution was adjusted to pH 4.0 with adding 1 M hydrochloric acid, followed by heating at 80 °C for 15 minutes, cooling to 15 °C, to thereby obtain about 200 ml of goat heated LF solution.

50 The denaturation rate of the LF contained in the solution was 99.7 % as measured by the same method as in Test 1 (liquid chromatography) .

## EFFECTS OF THE INVENTION

55 The effects of the present invention are as follows:

- 1) Heated LF prepared by the present invention can be safely used for foods, feeds, medicines and cosmetics, since it is a natural substance which is included in such as milk.
- 2) The method of present invention is suitable to be applied for preparation and processing, involving

heat treatment, of foods, feeds, medicines and cosmetics containing LF.

3) Any matters which contain LF can be sterilized, pasteurized or cooked by heating without affecting physiological activities of LF.

5 **Claims**

1. A method for heat treatment of lactoferrin in moisturized state or a matter containing moisturized lactoferrin, which comprises adjusting the pH of moisturized lactoferrin between 1.0 - 6.5 both inclusive, heating said matter at a temperature equal to or higher than 60 °C.

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2. A method as claimed in claim 1, wherein said heating is performed for a span of time which is determined depending upon the combination of conditions of a pH value between 1.0 - 6.5, a temperature equal to or higher than 60 °C and a relatively short time assuring an undenaturation rate of lactoferrin not less than 60 %.

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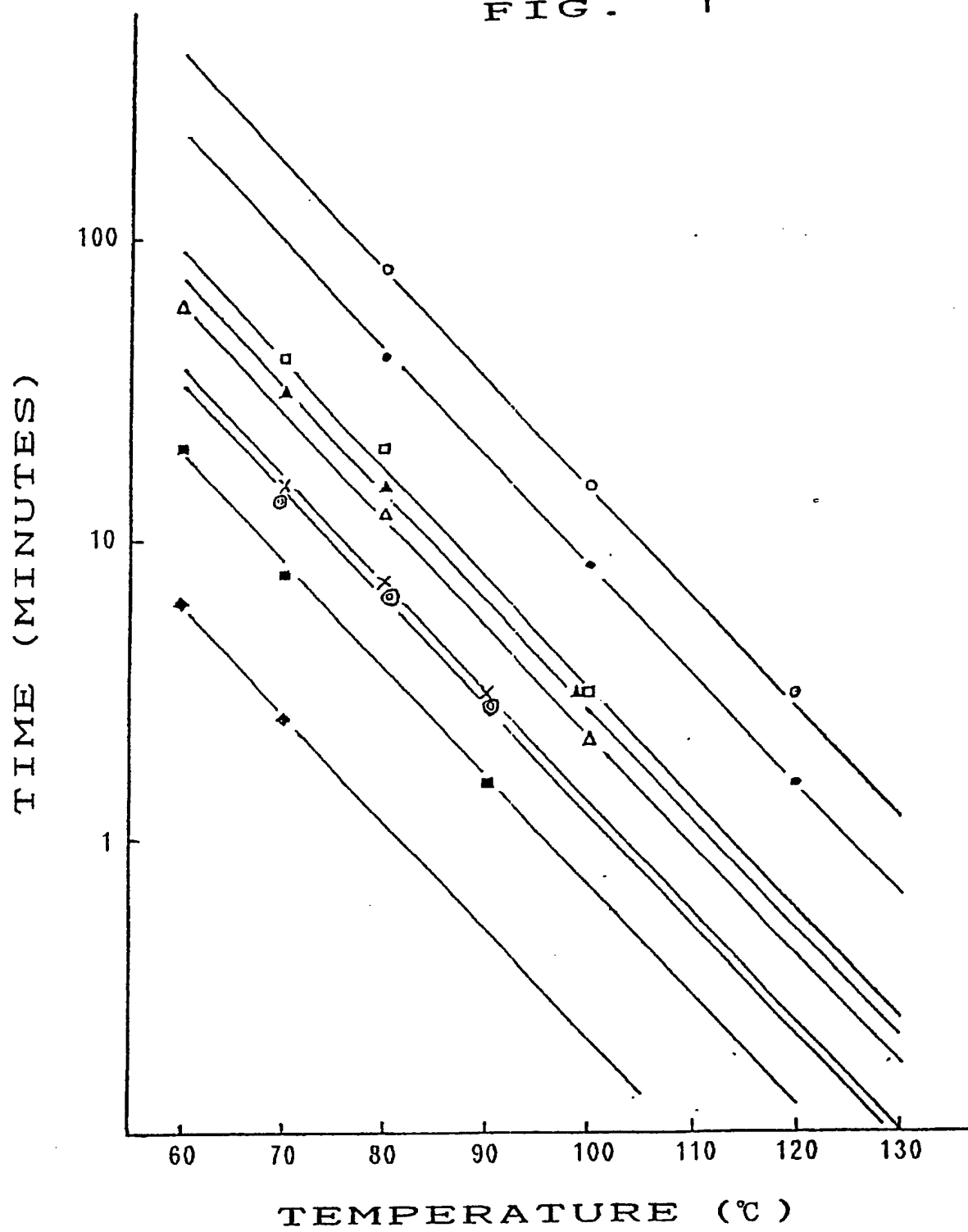
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FIG. 1





European  
Patent Office

## EUROPEAN SEARCH REPORT

Application Number

EP 90 31 3947

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	JOURNAL OF FOOD SCIENCE, vol. 51, no. 2, 1986, pages 348-351, Chicago, US; J.N. MORGAN et al.: "Thermal destruction of immunoglobulin A, lactoferrin thiamin and folic acid in human milk" * Pages 349-351, paragraph: "Discussion" * - - -	1-2	A 23 J 1/20
A	JOURNAL OF DAIRY SCIENCE, vol. 68, no. 11, 1985, pages 2847-2852, Cahmpaign, Illinois, US; V. BERNAL et al.: "Thermal stability of whey proteins - A calorimetric study" * Page 2847, paragraphs: "Abstract" and "Introduction"; pages 2849-2850, paragraph: "Results; PH" * - - - - -	1-2	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			A 23 J
The present search report has been drawn up for all claims			
Place of search The Hague		Date of completion of search 14 February 91	Examiner KORSNER S.E.
<div>CATEGORY OF CITED DOCUMENTS</div> <div>X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention</div> <div>E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons &amp;: member of the same patent family, corresponding document</div>			

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# Expression and Purification of Recombinant Rat Eosinophil-Associated Ribonucleases, Homologues of Human Eosinophil Cationic Protein and Eosinophil-Derived Neurotoxin, and Their Characterization

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## Key Words

Eosinophil · Eosinophil-associated ribonuclease-1 · Eosinophil-associated ribonuclease-2 · Bactericidal activity · Rat

## Abstract

**Background:** Human eosinophils contain two eosinophil ribonucleases, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). In rats, 8 homologues of human ECP and EDN have been identified. To clarify the biological activity of rat eosinophil ribonucleases, we cloned rat eosinophil-associated ribonuclease (EAR)-1/rat ribonuclease 7 and rat EAR-2/rat ribonuclease 4, and produced recombinant rat pre-EAR-1 and pre-EAR-2 in a bacterial expression system. **Methods:** As we have already cloned the complete nucleotide sequence for rat EAR-1, we determined that for rat EAR-2 cDNA by the rapid amplification of cDNA ends procedure. Recombinant rat pre-EAR-1 and pre-EAR-2 were expressed in *Escherichia coli* as N-terminal 6 × histidine-tagged proteins, isolated from the insoluble fraction of the cell lysate and purified by a single-step method using an Ni-NTA resin column after solubilization with a 6 M guanidine solution. **Results:** The deduced amino acid

sequence revealed that the molecular weight of EAR-2 containing the signal peptide is 17.3 kD and the isoelectric point is 8.59. The homology in amino acid sequence between rat pre-EAR-2, and human pre-ECP and human pre-EDN is 51 and 53%, respectively. The purified and refolded recombinant rat pre-EAR-1 and pre-EAR-2 showed bactericidal activity against *E. coli* and *Staphylococcus aureus*. **Conclusions:** These findings suggest that rat EAR-1 and EAR-2 act as host defense factors against bacterial infection in rats.

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## Introduction

Eosinophils are suggested to play a critical role in the pathogenesis of allergic diseases such as asthma [1–3] and atopic dermatitis [4, 5]. In these allergic diseases, eosinophils accumulate at the site of allergic inflammation and release granule proteins [1, 5]. It is widely accepted that the cytotoxic activity of these granule proteins plays a significant role in the pathogenesis of allergic inflammation by injuring the cells neighboring the infiltrating eosinophils.

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Human eosinophils contain four major proteins in granules: major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) [6]. ECP and EDN are members of the ribonuclease superfamily [7–9] and are similar in molecular mass ( $\approx 150$  amino acids) and in amino acid sequence ( $\approx 65\%$ ), although ECP is more cationic (pI 10.9) than EDN (pI 8.9) [7–9]. Human ECP is toxic to mammalian cells [10, 11] and has bactericidal and anti-helminthic activities [12–14], yet its ribonuclease activity is relatively weak [15–18]. In contrast, human EDN has little general toxicity [19], but has 50–100 times more ribonucleolytic activity than ECP [16, 17] and was reported to have ribonuclease-dependent antiviral activity [20–22]. The genes for ECP and EDN have been identified in humans [7–9] and other primates, including chimpanzees, gorillas, orang-utans, macaques and marmosets [23]. In mice Larson et al. [24] purified ribonucleases from mouse eosinophils and identified cDNA sequences of mouse eosinophil-associated ribonuclease (mEAR)-1 and mEAR-2 with approximately 50% amino acid sequence identity to human ECP and EDN. Batten et al. [25] identified another four related ribonuclease genes which form the mouse ribonuclease cluster together with mEAR-1 and mEAR-2.

In rats, we have already cloned cDNA for rat EAR-1 (rEAR-1) (accession No. D88586) [26], a homologue of human ECP and EDN. Recently, Shinghania et al. [27] identified eight rat ribonuclease (rR) genes that are the homologues of human ECP and EDN, although evolutionary analysis suggested no one-to-one pairing between any of the rat homologues and either of the known human genes. In the present study, we cloned cDNA for rEAR-1 (rR-7) and rEAR-2 (accession No. AB005291) (rR-4) from rat bone marrow cells, and expressed, purified, characterized and examined the bactericidal activity of recombinant rat EAR-1 and EAR-2 containing the signal peptide (pre-EAR-1 and pre-EAR-2, respectively).

## Materials and Methods

### *Preparation of Bone Marrow Cells from Rats Immunized with Ascaris suum Antigen*

Male rats of the Sprague-Dawley strain (specific pathogen-free, weighing 190–210 g, Charles River Japan, Kanagawa, Japan) received an oral administration of cyclophosphamide (Sigma, St. Louis, Mo., USA) suspended in 0.5% (w/v) sodium carboxymethylcellulose (Wako, Osaka, Japan) at a dose of 100 mg/kg on day 0. An aliquot (8 mg of protein) of *A. suum* extract (Greiner Laboratories, Lenoir, N.C., USA) as an antigen was suspended in 1 ml of saline containing 10 mg of aluminum hydroxide (Wako) as an adjuvant. On

day 2, as an immunization, 0.1 ml of the antigen suspension was injected intradermally at two nuchal and three lumbar sites, and 0.5 ml of the same solution was injected intraperitoneally. On day 12, an aliquot (5 mg of protein) of the antigen in 0.5 ml of saline containing 5 mg of aluminum hydroxide was injected intraperitoneally as a booster. Finally, on day 19, the rats were killed by cutting the carotid artery under diethylether anesthesia. The bone marrow cells were then collected from the shin bones and thigh bones. The cells were used after 3 washes with phosphate-buffered saline (PBS). The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan.

### *cDNA Cloning of Rat EAR-2*

The nucleotide sequence of rat EAR-2 was determined by RT-PCR and 5'- and 3'-rapid amplification of cDNA ends (RACE). From rat bone marrow cells, poly(A)<sup>+</sup> RNA was prepared using a QuickPrep mRNA Purification Kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 0.2  $\mu$ g of poly(A)<sup>+</sup> RNA by incubation at 37°C for 1 h in a 20 mM Tris-HCl buffer (pH 8.4) containing 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 5  $\mu$ M random hexamer oligonucleotides (Gibco-BRL, Gaithersburg, Md., USA), 200 units (U) of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), 0.5  $\mu$ M each of the deoxyribonucleoside 5'-triphosphates (dNTPs, Wako), 10 mM dithiothreitol and 100  $\mu$ g/ml of bovine serum albumin. For the determination of partial sequences of the EAR-2 gene, the PCR primers were designed from the amino acid sequence of rat EAR-1 as described previously [26]. The sequences of the primers were sense (5'-TGYYAARGGNATHAAYACNTT-3'), (5'-TTYGCNAAYGTTNGTGGNGT-3') and antisense (5'-GGRTTNACNCCNACNGT-RTA-3') (N = G, A, T or C; R = A or G; Y = T or C; H = A, T or C). A PCR was performed in 100  $\mu$ l of 10 mM Tris-HCl buffer (pH 8.3) containing 0.2  $\mu$ g of cDNA transcribed from poly(A)<sup>+</sup> RNA, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 250  $\mu$ M each of the dNTPs, 0.2  $\mu$ M each of the sense and the antisense primers, and 2.5 U of *Taq* polymerase (Takara Shuzo, Shiga, Japan), using a thermal cycler (GeneAmp PCR Systems 2400, Perkin Elmer Cetus, Norwalk, Conn., USA). The PCR cycle to amplify partial sequences of the rat EAR-2 gene consisted of 1 min of denaturation at 94°C, 1 min of annealing at 37°C, and 2 min of extension at 72°C for 35 cycles. The PCR products were purified with a low-melting agarose gel and ligated into the pGEM-T vector (Promega, Madison, Wisc., USA). The construct was verified by DNA sequence analysis using a Dyedeoxy Terminator Cycle Sequencing Kit (Perkin Elmer Japan Co., Urayasu, Chiba, Japan) and an Applied Biosystems Model 373A DNA Sequencer (Applied Biosystems, Foster City, Calif., USA). The nucleotide sequences of the 3'- and 5'-termini of the rat EAR-2 gene were determined by 3'- and 5'-RACE, respectively [28]. For 3'-RACE, poly(A)<sup>+</sup> RNA from the bone marrow cells of immunized rats was reverse transcribed using an oligo dT adaptor primer (5'-GTTTTCCAGTCACGAC (dT)<sub>20</sub>-3'). The PCR amplification was carried out using the primer (EAR-2 311–332 sense; 5'-ACCTGTAAAGACGGGAGAAGTA-3') as a sense primer and the adaptor primer (5'-GTTTTCCAGTCACGAC-3') as an antisense primer. The amplified 350-bp fragment was subcloned in the pGEM-T vector (Promega), and its nucleotide sequence was determined. For 5'-RACE, the poly(A)<sup>+</sup> RNA was reverse transcribed to generate the first-strand cDNA with the primer (EAR-2 410–429 antisense; 5'-CCTGTAGTTTGGTATCTGCA-3'). Addition of dATP to the newly synthesized DNA strand was accom-

plished using terminal deoxynucleotidyl transferase (Takara Shuzou). After the double-strand DNA was synthesized using the oligo dT adaptor primer, PCR amplification was carried out with the adaptor primer as a sense primer and the primer (EAR-2 372–391 antisense; 5'-CGGAGTTGTGAGGTTACAGA-3'). The amplified 400-bp fragment was subcloned in the pGEM-T vector (Promega), and its nucleotide sequence was determined.

#### *Construction of the 6 × Histidine-Tagged Protein Expression Vector for Pre-EAR-1 and Pre-EAR-2*

The cDNA for the open reading frame that encodes rat pre-EAR-1 and pre-EAR-2 protein was prepared by PCR using the following primers containing *Bam*HI (5') and *Sal*I (3') restriction sites (EAR-1: 5'-GGATCCATGGGTCTGAAGCTGCTC-3' (sense), 5'-GTCGACTACAAAGTGCTGGCACCG-3' (antisense), EAR-2: 5'-GGATCCATGGGTGTGAAGCCGCTT-3' (sense) and 5'-GTCGACGTACAAAGTGCTGGCATT-3' (antisense)) and, as templates, poly(A)+ RNA isolated from the bone marrow cells of immunized rats. The PCR cycle to amplify rat EAR-1 and EAR-2 genes consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 2 min of extension at 72°C for 35 cycles. The amplified 507-bp (EAR-1) and 501-bp (EAR-2) fragments were subcloned in the pGEM-T vector (Promega). The subcloned rat EAR-1 and EAR-2 cDNA were cleaved with *Bam*HI and *Sal*I, and the cDNA were excised. The cDNA fragments encoding rat pre-EAR-1 and pre-EAR-2 proteins were inserted into pQE-30 vector (Qiagen, Chatsworth, Calif., USA) and transformed in *E. coli* M15 (Qiagen) for the expression of 6 × histidine-tagged pre-EAR-1 and pre-EAR-2 proteins, respectively. The constructs were verified by DNA sequence analysis as described above.

#### *Expression and Purification of Recombinant Rat Pre-EAR-1 and Pre-EAR-2*

*E. coli* transfected with pQE-30-EAR-1 or pQE-30-EAR-2 vector was cultured at 37°C overnight in LB-broth (1% (w/v) Bacto-Tryptone (Difco Laboratories, Detroit, Mich., USA), 0.5% (w/v) Bacto-Yeast extract [Difco Laboratories], 85.5 mM NaCl) supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin. For the expression of recombinant rat pre-EAR-1 or pre-EAR-2, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Takara Shuzou) was added during the last 4 h of culture. After centrifugation at 1,400 g and 4°C for 30 min, the *E. coli* was washed 3 times with PBS, suspended in PBS containing 0.5% Triton X-100 and sonicated. The cell pellet obtained by centrifugation at 1,400 g and 4°C for 30 min was solubilized with buffer A (pH 8.0, 10 mM Tris-HCl, 6 M guanidine, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM 2-mercaptoethanol and 20 mM imidazole) by stirring for 1 h at room temperature. To remove the cellular debris, the suspension was centrifuged at 10,000 g and 20°C for 20 min, and a solubilized intracellular insoluble fraction was obtained. Purification of recombinant rat pre-EAR-1 and pre-EAR-2 was carried out using an Ni-NTA resin column (Qiagen). The solubilized intracellular insoluble fractions were applied to the Ni-NTA resin column equilibrated with buffer A, washed with buffer A and buffer B (pH 6.3, 10 mM Tris-HCl, 8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM 2-mercaptoethanol, 20 mM imidazole). Recombinant rat pre-EAR-1 and pre-EAR-2 were eluted with buffer C (pH 6.3, 10 mM Tris-HCl, 8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM 2-mercaptoethanol, 250 mM imidazole).

#### *Refolding of the Fusion Protein*

Recombinant rat pre-EAR-1 and pre-EAR-2 eluted from the Ni-NTA resin column were refolded by phased-urea-removing dialysis with 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl (pH 6.3) and urea. The concentration of urea in the dialysis buffer was sequentially decreased (4, 1, 0.5, 0.1, 0.01 and 0 M). Precipitates developing during dialysis were removed by centrifugation at 18,600 g and 4°C for 20 min and the supernatant was used as a sample for the test.

#### *SDS-PAGE Analysis*

Samples reduced with 10% 2-mercaptoethanol in 50 mM Tris-HCl buffer (pH 6.8) containing 1% sodium dodecylsulfate (SDS) and 20% glycerol were applied to a 15% (w/v) SDS-polyacrylamide gel. The proteins separated in the gel were stained using a Silver Stain II Kit (Wako).

#### *Growth Curves of E. coli*

Overnight cultures of *E. coli* transfected with pQE-30-EAR-1, pQE-30-EAR-2 or pQE-30 vector alone were diluted 30-fold, in LB-broth containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The transformants were incubated at 28°C. Optical densities at 600 nm were recorded by a UV-1200 Spectrophotometer (Shimadzu, Japan) at 2-hour intervals for 8 h. After 4 h of incubation, IPTG (final 0.2 mM) was added to the cultures.

#### *Determination of Bactericidal Activity of Recombinant Rat Pre-EAR-1 and Pre-EAR-2*

Bactericidal activities of the purified proteins against *E. coli* LE392 (Funakoshi, Tokyo, Japan) and *S. aureus* Cowan I (Institute of Medical Sciences, Tokyo University, Tokyo, Japan) strains were determined according to the procedure described by Lehrer et al. [14]. The bacteria were maintained on LB-agar plates. A single colony of bacteria was inoculated into 10 ml of LB-broth and cultured for 16 h at 37°C. Then, 0.1 ml of this intermediate culture was diluted with 5 ml of fresh nutrient and incubated for an additional 2 h at 37°C. A portion of this mid-logarithmic phase culture was washed with 10 mM sodium phosphate buffer (pH 7.4), and  $1 \times 10^7$  bacteria were incubated with various concentrations of the purified protein in 10 mM Tris-HCl (pH 6.3) containing 0.1 M NaH<sub>2</sub>PO<sub>4</sub> and 1% (v/v) LB-broth. After incubation for 2 h at 37°C, 0.1 ml of the sample was diluted with 10 mM sodium phosphate buffer (pH 7.4), serially spread on nutrient agar plates and incubated for 12 h at 37°C to allow full colony development. Then the colonies on each plate were enumerated and the number of bacterial colony-forming units was calculated [14].

#### *Statistical Analysis*

The statistical significance of the results was analyzed by Dunnett's test for multiple comparison.

## **Results**

#### *cDNA Cloning of Rat EAR-2*

Poly (A)+ RNA was obtained from bone marrow cells of rats immunized with *A. suum* antigen and was used as a template for RT-PCR to determine the partial sequence of cDNA for rat EAR-2. We first performed PCR amplifi-

```

1      TGATTTCAGGACAGCCAGCACTCAGTTCCACAGGAGCCACAAACGACACTGGGAAAC
59  ATG GGT GTG AAG CCG CTT GAG TCC AGA CTT TGT CTC CTT CTG CTG CTG GGA CTT GTC TCA
1   M   G   V   K   P   L   E   S   R   L   C   L   L   L   L   L   G   L   V   S
119  ATG CTT GCC TCA TGC CAG CGA CCA ACC OCT TCC CAG TGG TTT GCC ATC CAG CAC ATC TAT
21   M   L   A   S   C   Q   R   P   T   P   S   Q   W   F   A   I   Q   H   I   Y
179  AAT AGC TCC TAC CCC CAA TGT AAT GCT GCC ATG CTG CGT GTC AAC AGT TAT ACA GGA AGA
41   N   S   S   Y   P   Q   C   N   A   A   M   L   R   V   N   S   Y   T   G   R
239  TGT AAG GGC ATA AAT ACT TTT CTT CAC ACA AGT TTT GCT AGT GTT GTT GAT GTG TGT GGC
61   C   K   G   I   N   T   F   L   H   T   S   F   A   S   V   V   D   V   C   G
299  AAT CCA CAT ATA ACC TGT AAA GAC GGG AGA AGT ACA AAT TGT CAT AAC AGT TCC TCT CAG
81   N   P   H   I   T   C   K   D   G   R   S   T   N   C   H   N   S   S   S   Q
359  GTA TCT GTA ACT TTC TGT AAC CTC ACA ACT CCG GCA AGG ATT TAT ACC CAA TGC AGA TAC
101  V   S   V   T   F   C   N   L   T   T   P   A   R   I   Y   T   Q   C   R   Y
419  CAA ACT ACA GGA TCG GTG AAG TTC TAC AGA GTT GCC TGT AAC AAT AGA ACT TCA CAG GAG
121  Q   T   T   G   S   V   K   F   Y   R   V   A   C   N   N   R   T   S   Q   E
479  AGT CCC ATG TAT CCA GTG GTT OCT GTT CAC CTG GAT GAG ATA TTT TAG TTCAATGCCAGCACT
141  S   P   M   Y   P   V   V   P   V   H   L   D   E   I   F   *   155
542  TTGTACCTTAGCTCATCTGCTACTGCGCAAGATCACAGTAGTGAAGATCCAGTTCTCTGTCTGCACTGCTAACCAGACC
621  TTTTCTAACCAATAAACACCTTTGCATTCAAAAAAA 658

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**Fig. 1.** cDNA and deduced amino acid sequences of rat EAR-2. Potential N-linked glycosylation sites are underlined and the polyadenylation signal is double-underlined. A signal peptide is shown by a dotted line. The presumptive stop codon (TAG) is shown by an asterisk. The numbers of nucleotides and amino acids are indicated in the left margin.

cation using the three degenerate primers based on the amino acid sequence of rat EAR-1, as described previously [26]. When PCR was carried out using certain primers, a 190-bp nucleotide fragment was strongly amplified (data not shown). The 190-bp fragment was then subcloned in a plasmid vector, and the complete nucleotide sequence was determined. The nucleotide sequence of the 190-bp fragment was highly homologous to that of the human ECP (70% homology, data not shown). Therefore, we determined the cDNA sequence of the full coding region of the protein by the RACE procedure based on the sequence of the 190-bp PCR fragment. By 3'- and 5'-RACE, 350- and 400-bp fragments were amplified, respectively. The nucleotide sequences of the 350- and the 400-bp fragments were found to encode the 3'- and 5'-termini of cDNA for rat EAR-2, respectively. Because the cloned gene is more homologous to the genes of mEARs than to those of human and primate ECP and EDN, we named it rat EAR-2 gene, and found that this gene is identical with rat ribonuclease 4 reported by Shinghania et al. [27].

The complete nucleotide sequence of rat EAR-2 contains 658 bases, and the polyadenylation signal is an

AATAAA-type at nucleotides 632–637 (fig. 1). The number of amino acids in the open reading frame of rat pre-EAR-2 is 155; the molecular weight of the protein is 17.3 kD. The N-terminal amino acid of mature rat EAR-2 was presumed to be Ser<sup>24</sup> as described by Heijne [29]. The pI values of rat pre-EAR-2 (Met<sup>1</sup> to Phe<sup>155</sup> in fig. 1) and mature EAR-2 (Ser<sup>24</sup> to Phe<sup>155</sup> in fig. 1) were calculated to be 8.59 and 8.52, respectively.

Figure 2 shows the alignment of the deduced amino acid sequence of rat EAR-1 [26] and EAR-2 containing the signal peptide (Met<sup>1</sup> to Ala<sup>23</sup>) and that of human ECP [7, 8] and EDN [9]. The homology in amino acid sequence between rat pre-EAR-2, and human pre-ECP, human pre-EDN, mouse pre-EAR-1 and mouse pre-EAR-2 is 51, 53, 65, and 65%, respectively. The homology between rat pre-EAR-2 and rat pre-EAR-1 is 74%.

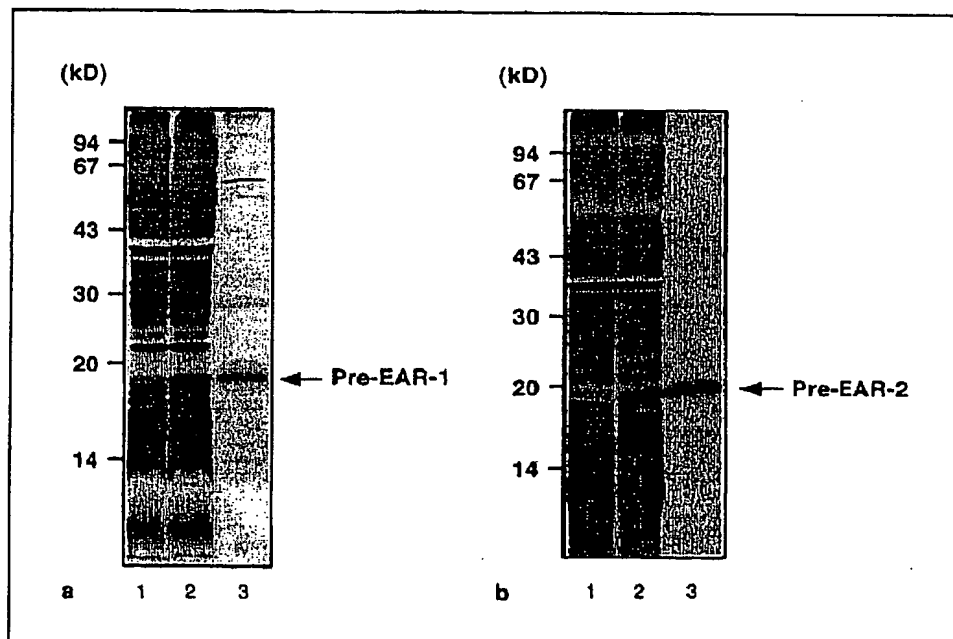
#### *Production and Purification of Recombinant Rat Pre-EAR-1 and Pre-EAR-2*

Recombinant rat pre-EAR-1 and pre-EAR-2 were expressed as N-terminal 6 × histidine-tagged proteins in *E. coli*, and were purified from the lysate of *E. coli* using the Ni-NTA resin column under denaturing conditions. The

Rat Pre-EAR-1	MGLKLLSRICLLLSLGL--V--LML-ASC-QPPTPSQWFEIQH <sup>*</sup> YINRAYPCNDA	50
Rat Pre-EAR-2	..V.P.....L.....S.....R.....A.....SS..Q..A.	50
Human Pre-ECP	.VP..FT.QI....L...MG.EGS-.H.RPP.F-.RA...A...SLNPP-.TI.	57
Human Pre-EDN	.VP..FT.QI....L...LA.EGS-.HVKPP.F-.WA.....NMISQ-Q.TN.	57
Rat Pre-EAR-1	MRHRNRFTGHCKDINTFLHTSPASVVGVCGRNIPCG-NRTYRNCHNSRYRVSITFCNLIT	110
Rat Pre-EAR-2	.LRV.SY..R..G.....D....PH.T.KDG.S-T.....SSQ..V.....	110
Human Pre-ECP	..AI.NYKWR..NQ....R.T..N..N....QS.R.PH...LN...R..P..PLH.D.IN	108
Human Pre-EDN	.QVI.NYQRR..NQ....L.T..N..N....P.MT.PS.K.RK...H.GSQ.PLIH....	108
Rat Pre-EAR-1	P-ARIYTQCRVQTTRSRKFTYVGCDPRT-PRDSPMXPVVPVHLDRIF	155
Rat Pre-EAR-2	.....G.V...R.A.NN.-SQE.....E..	155
Human Pre-ECP	.G.QNISN...ADRPGR..V.A..N.D.....R.....TTI	154
Human Pre-EDN	.SPQNISN...AQ.PANM..I.A..N.DQR..P.Q.....I	155
		pI
		9.9
		8.6
		10.5
		8.7

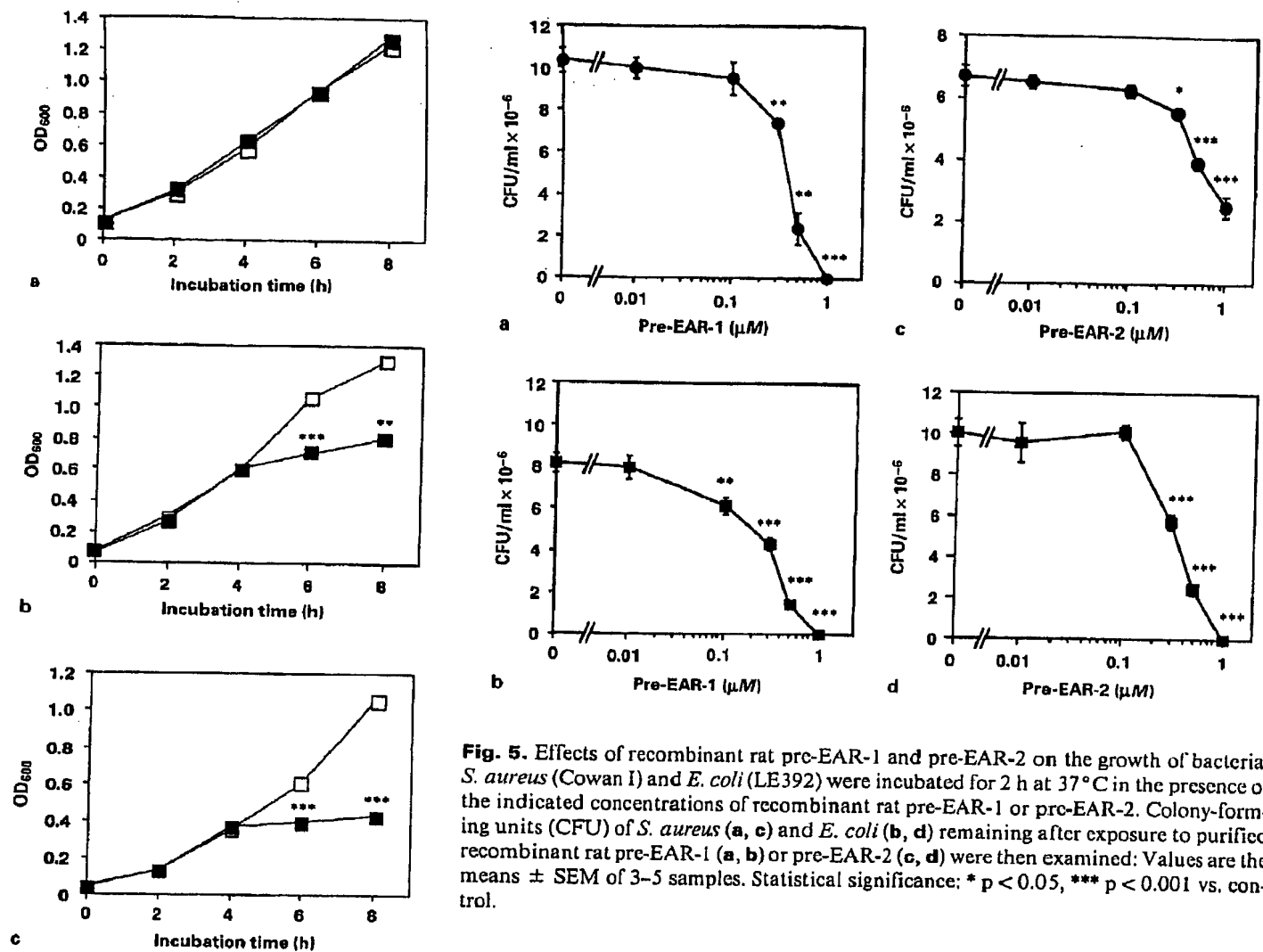
**Fig. 2.** Amino acid sequences of rat pre-EAR-1 and pre-EAR-2 and comparison with human pre-ECP and human pre-EDN. The amino acid sequences deduced from the cloned cDNA for rat EAR-1 and EAR-2 are compared with those of human ECP and human EDN. The numbers of amino acids are indicated in the right margin. Dots show the same amino acids as those of the sequence in rat pre-EAR-1. Gaps have been introduced to achieve maximum sequence homology. \* Required for RNase activity; # structural Cys.

**Fig. 3.** SDS-PAGE analysis of the purified recombinant rat pre-EAR-1 and pre-EAR-2. The recombinant rat pre-EAR-1 and pre-EAR-2 were expressed by *E. coli* transfected with the pQE-30-EAR-1 or pQE-30-EAR-2 constructs and purified in an Ni-NTA resin column. Lysates from *E. coli* and the purified proteins were subjected to SDS-PAGE analysis. Lanes 1 and 2 contain total cell extracts from *E. coli* transfected with the pQE-30-EAR-1 (a) or pQE-30-EAR-2 (b) constructs prior to (lane 1) and 4 h after addition of 0.2 mM IPTG (lane 2). Lane 3 contains recombinant rat pre-EAR-1 or pre-EAR-2 purified in an Ni-NTA resin column.



results of SDS-PAGE (15% gel) analysis of proteins obtained from *E. coli* transfected with pQE-30-EAR-1 or pQE-30-EAR-2 at different steps of expression and purification are shown in figure 3. By the addition of IPTG, an 18-kD protein was induced (lane 2). The 18-kD protein

was detected in neither the flow-through fraction nor the washing fraction from the Ni-NTA resin column (data not shown). Recombinant rat pre-EAR-1 (fig. 3a) and pre-EAR-2 (fig. 3b) were detected (lane 3) in the eluate from the Ni-NTA resin column.



**Fig. 5.** Effects of recombinant rat pre-EAR-1 and pre-EAR-2 on the growth of bacteria. *S. aureus* (Cowan I) and *E. coli* (LE392) were incubated for 2 h at 37°C in the presence of the indicated concentrations of recombinant rat pre-EAR-1 or pre-EAR-2. Colony-forming units (CFU) of *S. aureus* (a, c) and *E. coli* (b, d) remaining after exposure to purified recombinant rat pre-EAR-1 (a, b) or pre-EAR-2 (c, d) were then examined. Values are the means  $\pm$  SEM of 3–5 samples. Statistical significance: \*  $p < 0.05$ , \*\*\*  $p < 0.001$  vs. control.

**Fig. 4.** Effects of the expression of rat pre-EAR-1 and pre-EAR-2 on the growth of *E. coli*. *E. coli* (M15) was transfected with empty vectors (pQE-30) (a), expression plasmid vectors of pre-EAR-1 (pQE-30-EAR-1) (b) or pre-EAR-2 (pQE-30-EAR-2) (c). Each transformant was incubated at 28°C. IPTG (final 0.2 mM) was added to cultures after 4 h of incubation. □ = Growth of *E. coli* without IPTG treatment; ■ = growth of *E. coli* with IPTG treatment. Values are the means of 3 samples. The SEM lie within the symbols. Statistical significance: \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. condition without IPTG.

#### Effects of the Expression of Rat Pre-EAR-1 and Pre-EAR-2 on the Growth of *E. coli*

Effects of the expression of rat pre-EAR-1 and pre-EAR-2 on the growth of *E. coli* (M15) were examined. After 4 h of incubation, IPTG (0.2 mM) was added to the

cultures of *E. coli* transfected with pQE-30 vector alone, pQE-30-EAR-1 or pQE-30-EAR-2. The growth of *E. coli* transfected with pQE-30 vector alone was unaffected by IPTG (fig. 4a), but the growth of *E. coli* transfected with pQE-30-EAR-1 or pQE-30-EAR-2 was inhibited by the addition of IPTG to the cultures (fig. 4b, c).

#### Bactericidal Activity of Recombination Rat Pre-EAR-1 and Pre-EAR-2

We examined whether the recombinant rat pre-EAR-1 and pre-EAR-2 have bactericidal activity. *S. aureus* (Cowan I) and *E. coli* (LE392) were incubated for 2 h at 37°C in the presence of various concentrations of recombinant rat pre-EAR-1 (fig. 5a, b) and pre-EAR-2 (fig. 5c, d). Growth of both *S. aureus* and *E. coli* was inhibited by recombinant rat pre-EAR-1 and pre-EAR-2 in a concen-

tration-dependent manner. Pre-EAR-1 at 1  $\mu\text{M}$  almost completely inhibited the growth of *S. aureus* and *E. coli* (fig. 5a, b); pre-EAR-2 at 1  $\mu\text{M}$  did not completely inhibit the growth of *S. aureus* (38% of the colony-forming units remained) although it almost did with *E. coli* at 1  $\mu\text{M}$  (fig. 5c, d).

## Discussion

Previously, we have characterized the rat homologue of human ECP and EDN, rat EAR-1 (accession No. D88586; the DDBJ, EMBL and NCBI nucleotide sequence databases) [26] from rat bone marrow cells. In the present study, we have cloned cDNA for rat EAR-2 (accession No. AB005291) (rat ribonuclease 4) encoding another rat homologue of human ECP and EDN. Because the nucleotide sequences of rat EAR-1 and EAR-2 are more homologous to those of mouse EARs [24] than to those of human ECP and EDN, we named the cloned genes 'rat EAR-1' and 'rat EAR-2' cDNA, respectively. Recently, Shinghania et al. [27] identified eight rat ribonuclease genes including rat EAR-1 [26] and EAR-2, and suggested by evolutionary analysis that mouse EARs and rat EARs have evolved independently in mice and rats. In humans, ECP shows more potent anti-helminthic [19] and bactericidal activity [30] than EDN, but EDN possesses 50–100 times more ribonuclease activity than ECP [16, 17]. Rosenberg [30] reported that the granule-derived human ECP at 1.15  $\mu\text{M}$  reduced the colony formation of *S. aureus* by more than 90%, and the recombinant human ECP at 1  $\mu\text{M}$  reduced it by more than 60%. He also reported that neither the granule-derived human EDN at 5.5  $\mu\text{M}$  nor recombinant human EDN at 1  $\mu\text{M}$  have bactericidal activity [30]. We have already reported that rat EAR-1 purified from rat eosinophils reduces colony formation of *E. coli* and *S. aureus* by more than 99% at 0.2  $\mu\text{M}$  [31]. To clarify the biological properties of rat EARs, we have produced recombinant rat pre-EAR-1 and pre-EAR-2 in a bacterial expression system and have shown that both purified and resolubilized recombinant rat pre-EAR-1 and pre-EAR-2 from bacterial inclusion bodies show bactericidal activity against *S. aureus* and *E. coli*.

When the amino acid sequence of rat pre-EAR-2 was compared with that of human pre-ECP and pre-EDN, 3 amino acids (His<sup>38</sup>, Lys<sup>62</sup>, His<sup>150</sup>) that are required for RNase activity [7, 30, 32] and 8 structural cysteines were found to be highly conserved (fig. 2). It is reported that the substitution of Lys<sup>38</sup> and His<sup>128</sup> in human mature ECP [30] and of Lys<sup>38</sup> in human mature EDN [32] for other

amino acids diminished the RNase activity. Like rat pre-EAR-1 [26], rat pre-EAR-2 also has two histidines (His<sup>38</sup>, His<sup>150</sup>) and one lysine (Lys<sup>62</sup>) (fig. 2). Since these amino acids are reported to be essential for RNase activity in mice [24] and humans [30, 32] at such positions, rat EAR-1 and EAR-2 may also have RNase activity.

Recombinant rat pre-EAR-1 and pre-EAR-2 were designed to be expressed as N-terminal 6  $\times$  histidine-tagged proteins in *E. coli*. The 6  $\times$  histidine-affinity-tag facilitates binding to Ni-NTA, and is weakly immunogenic. The tag is also small in size and uncharged at pH 8.0. In most cases, the 6  $\times$  histidine-tag does not interfere with the structure or the function of the purified protein as demonstrated for a wide variety of proteins, including enzymes [33], transcription factors [34] and vaccines [35]. Recombinant rat pre-EAR-1 and pre-EAR-2 were expressed in *E. coli* as insoluble inclusion bodies, solubilized with 6 M guanidine solution and purified by a simple one-step method using Ni-NTA resin. The eluted protein was refolded by successively decreasing the urea concentration by dialysis. The recombinant protein has a predicted molecular size as revealed by SDS-PAGE analysis (fig. 3).

It is reported that the granule-derived human ECP and the recombinant human ECP have bactericidal activity against *S. aureus* and *E. coli* [30, 32]. We have previously reported that the granule-derived rat EAR-1 also showed strong bactericidal activity against *S. aureus* and *E. coli* [31] under the conditions described by Lehrer et al. [14]. However, it was unclear whether the granule-derived rat EAR-2 has bactericidal activity. Therefore, we examined the bactericidal activity of the purified recombinant rat pre-EAR-2. The biological activities of human eosinophil granule proteins are due to their highly basic characteristics [36]. In rat EARs, there is little difference in the pI values between pre-EARs and mature EARs (the pI values are: pre-EAR-1, 9.85; mature EAR-1, 9.87; pre-EAR-2, 8.59; mature EAR-2, 8.52). Therefore, in the present study, we examined the bactericidal activity of preforms of EAR-1 and EAR-2.

As shown in figure 5, both recombinant rat pre-EAR-1 and pre-EAR-2 inhibited the growth of *S. aureus* and *E. coli* in a concentration-dependent manner, indicating that recombinant rat pre-EAR-1 and pre-EAR-2 possess bactericidal activity. Rosenberg [30] reported that granule-derived human EDN has no bactericidal activity at concentrations where granule-derived human ECP shows bactericidal activity, and confirmed the results using recombinant proteins. The mechanism by which human ECP (pI 10.8) exerts its bactericidal activity has not been

clarified, but it has been proposed that its cationic residues might disrupt membrane phospholipid bilayers [10]. The lack of bactericidal activity of the granule-derived human EDN [30] might be due to its lower pI value (pI 8.9). But recombinant rat pre-EAR-2 showed bactericidal activity in our system (fig. 5c, d), although its pI value is 8.6. This finding suggests that a net positive charge is not essential for bactericidal activity.

To summarize, we cloned cDNA encoding rat EAR-2 (rR-4), a homologue of human ECP and EDN. We prepared recombinant rat pre-EAR-1 and pre-EAR-2 using a bacterial expression system, and found that the purified recombinant rat pre-EAR-1 and pre-EAR-2 showed bac-

tericidal activity. Clarification of the classes of animals that have eosinophil RNase might be important for interpreting the origin of eosinophil in animals and understanding the functions of eosinophils in host defense systems.

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